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Original Research

Targeted Next Generation Sequencing molecular profiling and its clinical application in adrenocortical cancer

Francesca Cioppi,[1,2,*†] Giulia Cantini,[2,3,4,†] Tonino Ercolino,[5] Massimiliano Chetta,[6] Lorenzo Zanatta,[2,3,5] Gabriella Nesi,[7] Massimo Mannelli,[2,3,4] Mario Maggi,[2,3,4,5] Letizia Canu,[2,3,4,5] and Michaela Luconi[2,3,4,*]

[1]Department of Experimental and Clinical Medicine, University of Florence, 50139 Florence, Italy
[2]European Network for the Study of Adrenal Tumours (ENSAT) Centre of Excellence, University of Florence, 50139 Florence, Italy
[3]Department of Experimental and Clinical Biomedical Sciences, Endocrinology Section, University of Florence, 50139 Florence, Italy
[4]Centro di Ricerca e Innovazione sulle Patologie Surrenaliche, AOU Careggi, 50139 Florence, Italy
[5]Azienda Ospedaliero-Universitaria Careggi, (AOUC), 50139 Florence, Italy
[6]Medical Genetics, Azienda Ospedaliera di Rilievo Nazionale (A.O.R.N.) Cardarelli, Padiglione, 80131 Naples, Italy
[7]Department of Health Sciences, University of Florence, 50139 Florence, Italy

*Corresponding author: Department of Experimental and Clinical Medicine, University of Florence, viale Pieraccini 6, Florence 50139, Italy. Email: francesca.cioppi@unifi.it; Department of Experimental and Clinical Biomedical Sciences, University of Florence, viale Pieraccini 6, Florence 50139, Italy. Email: michaela.luconi@unifi.it

Abstract

Objective: Adrenal cortical carcinoma (ACC) is a rare malignancy with a generally poor but heterogeneous prognosis, especially depending on the tumour stage at diagnosis. Identification of somatic gene alterations combined with clinical/histopathological evaluation of the tumour can help improve prognostication. We applied a simplified targeted-Next-Generation Sequencing (NGS) panel to characterise the mutational profiles of ACCs, providing potentially relevant information for better patient management.

Design and methods: Thirty frozen tumour specimens from a local ACC series were retrospectively analysed by a custom-NGS panel (*CDKN2A, CTNNB1, DAXX, MED12, NF1, PRKAR1A, RB1, TERT, TP53, ZNRF3*) to detect somatic prioritised single-nucleotide variants. This cohort was integrated with 86 patients from the ACC-TCGA series bearing point-mutations in the same genes and their combinations identified by our panel. Primary endpoints of the analysis on the total cohort (113 patients) were overall survival (OS) and progression-free survival (PFS), and hazard ratio (HR) for the different alterations grouped by the signalling pathways/combinations affected.

Results: Different PFS, OS, and HR were associated to the different pathways/combinations, being *NF1 + TP53* and Wnt/β-catenin + Rb/p53 combined mutations the most deleterious, with a statistical significance for progression HR which is retained only in low-(I/II) stages—*NF1 + TP53* combination: HR = 2.96[1.01-8.69] and HR = 13.23[3.15-55.61], all and low stages, respectively; Wnt/β-catenin + Rb/p53 combined pathways: HR = 6.47[2.54-16.49] and HR = 16.24[3.87-68.00], all and low-stages, respectively.

Conclusions: A simplified targeted-NGS approach seems the best routinely applicable first step towards somatic genetic characterisation of ACC for prognostic assessment. This approach proved to be particularly promising in low-stage cases, suggesting the need for more stringent surveillance and personalised treatment.

Keywords: ACC, somatic mutational profile, routine analysis, progression-free survival, genetics, targeted-NGS, molecular oncology, ACC-TCGA

Significance

Several genes have been identified as recurrently mutated in ACC tumours, offering potential DNA-based biomarkers for clinical outcomes. However, tumour molecular profiling has not yet been introduced in routine management of ACC. Starting from a small monocentric local cohort then integrated with data from ACC-TCGA patients, we demonstrated that a light custom targeted-NGS panel, including only those genes clinically relevant in ACC provided prognostication ability, especially in patients diagnosed with low-risk disease on the basis of the tumour stage. These findings underline how molecularly guided patient stratification using NGS analysis in everyday clinical practice may improve individualised treatment strategies.

Introduction

Adrenal cortical carcinoma (ACC) is a rare endocrine malignancy originating from the adrenal cortex, with an annual worldwide incidence of .5-2:1 million[1,2] and a bimodal age distribution.[3,4] Approximately 50%-60% of cases display clinical evidence of hormonal excess, mainly hypercortisolism.[5,6] This tumour behaves aggressively and survival rate is poor, particularly when it is diagnosed at advanced stages.[5,7] Prognosis is heterogeneous and pathological stage proves to be the main prognostic factor[7] along with the proliferation index Ki67,[8,9]

† F.C. and G.C. contributed equally.

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possibly implemented by the S-GRAS score, which in addition to Ki67 and stage, includes age, tumour resection status, and symptoms as independent prognostic factors.[10] Cortisol excess[11] and Weiss score parameter prioritisation[12] can be considered additional predictive factors. Surgery is the only curative treatment, although local recurrence and metastases are common, even after resection with negative margins.[5] In the adjuvant setting, mitotane monotherapy in high-risk patients (stage III and Ki67 > 10%),[13] or with etoposide–doxorubicin–cisplatin (EDP) in unresectable cases is recommended.[14,15]

ACC molecular classification on the basis of somatic alterations in the cancer genome may integrate clinico-pathological prognosticators, and help predict individual outcomes while supporting the development of more effective and personalised therapies. In recent years, understanding of ACC genetics has improved, leading to the identification of driver genes involved in the pathogenesis of this malignancy. Two large pan-genomic studies have shown several recurrently altered genes in the tumour of ACC patients, including *CDKN2A, CTNNB1, DAXX, MED12, MEN1, NF1, PRKAR1A, RB1, TERT, TP53,* and *ZNRF3*[16,17] validated as the major ACC driver genes in omics-based independent studies, integrating expression, epigenetic, miRNA, chromosomal, copy-number variation (CNV) profiles.[18,19] Since genome-wide analysis is time-consuming, expensive, and generates a sizable quantity of complex data, a targeted-Next-Generation Sequencing (NGS) approach is advisable. Targeted-sequencing is now an important routine technique in both clinical and research settings, offering advantages (acceptable turnaround times, low costs, fewer computational burden, high confidence and accuracy[20]).

Here, we assessed the ability of a custom targeted-NGS panel, consisting of 10 of the most frequently mutated genes in ACC and applied to a small local cohort of ACC patients, to identify the mutation profiles. Integration of the local cohort with data from ACC-TCGA allowed the evaluation of the identified point mutations in a prognostic factor analysis, increasing the prognostication power, in particular in low-stage patients, generally considered at low risk of survival.

Materials and methods

Local cohort patients

This study was designed and conducted in accordance with the Declaration of Helsinki. We retrospectively analysed a local series of 30 conventional primary ACCs operated at Careggi University Hospital (European Network for the Study of Adrenal Tumours-ENSAT Centre of Excellence), between 2000 and 2021. A formal sample size calculation was not performed due to the rarity of the tumour. All suspected lesions underwent laparoscopic surgery according to ENSAT ACC Guidelines.[5] The study was approved by the local Ethical Committee (Prot. 2011/0020149), and recruited patients gave their written informed consent. At surgery, resected specimens were formalin-fixed and paraffin-embedded (FFPE) or snap frozen. Blood samples were drawn before surgery for Sanger sequencing of variants on germline DNA. The characteristics of the local series are indicated in Table 1.

Pathological analysis of ACC samples from the local cohort

Histological diagnosis was carried out by two independent reference pathologists on the tumour tissue removed at surgery. Tumour specimens were evaluated according to the Weiss scoring system.[21] The Ki67 labelling-index (LI) was estimated using the anti-human Ki67 antibody (1:40 dilution, MIB-1, Dako, CA, USA).[22] Ki67-positive nuclei were counted in 1000 tumour cells, and Ki67-LI expressed as labelled-cell percentage. Tumour stage was assessed according to ENSAT classification.[23] Tumour margin status is expressed as no residual tumour (R0), microscopic residual tumour (R1), macroscopic residual tumour (R2), uncertain resection status (Rx).

DNA extraction from ACC specimens and blood samples

Patient genomic DNA was extracted from the local cohort of 30 frozen tumour specimens and blood samples using DNeasy Blood & Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA quality and quantity were measured by the Qubit ds assay (Thermo Fisher Scientific, MA, USA).[24]

Gene panel design and sequencing

A novel custom targeted-panel of 10 genes (Table 2), previously identified as driver genes for ACC,[15,16] was created with SureDesign software (Agilent Technologies, UK), encompassing a targeted region of 115.17 kpb utilising 891 amplicons with mean sequence coverage = 98.53 at 20× priori coverage of 99.41%, on the basis of our expertise in designing targeted-NGS panels.[27,28] Library amplification and variant filtering are detailed in Data. The SNVs obtained were manually filtered as in Figure 1A.[29]

Protein folding *in silico* analysis

Modifications in protein tertiary structure were analysed using Phyre2 software (Protein Homology/analogY Recognition Engine V 2.0 Server; www.sbg.bio.ic.ac.uk/phyre2/) to assess the potential functional effect of VUS observed in *TERT, ZNRF3, PRKAR1A* and *RB1* genes. The 3D-structures of wild type and mutant proteins generated by Phyre2 were compared and displayed with Chem3D 20.1.1.125 (Cambridge Software, PerkinElmer, Inc., Massachusetts, USA).

ACC-TCGA cohort

From the ACC-TCGA cohort,[17] we selected 86 conventional ACCs patients (https://www.cbioportal.org,[30]) including only those patients with or without point mutations in those genes found altered in more than one patient in the local cohort (*CTNNB1, ZNRF3, NF1, TP53, RB1* genes). We limited our query to *CTNNB1, ZNRF3, NF1, TP53, RB1* genes; *TERT* was not considered since the alterations reported in the ACC-TCGA were promoter amplification instead of point mutations in the encoding sequence. We also excluded *PRKAR1A* as low represented and never alone. All these exclusion criteria have been adopted in order to limit any variability between the two cohorts in terms of candidate point mutations and their combinations. Clinical and pathological characteristics extracted from https://www.cbioportal.org and chemotherapy information derived from https://portal.gdc.cancer.gov/exploration are listed in Table 3.

Statistical analysis

Statistical analysis was performed with SPSS28.0 for Windows (Chicago, IL, USA). Continuous variables with normal distribution were presented as mean ± standard deviation (SD) and nonparametric variables as median [interquartile range

Table 1. Clinical characteristics of the local monocentric cohort of 30 ACC patients.

	ACC Local Cohort	*N* = 30	WT	*N* = 13	Patients with Mutation/Variant	*N* = 17
Age (years)	50 ± 11	30/30	50 ± 10	13/13	51 ± 12	17/17
Male sex (%)	11 (37)	30/30	7 (54)	13/13	4 (24)	17/17
Secretion (%)		28/30		13/13		15/17
Cortisol	12 (40)		7 (54)		5 (29)	
Androgens	6 (20)		2 (15)		4 (24)	
Aldosterone	1 (3)		0 (0)		1 (6)	
NS	9 (30)		4 (31)		5 (29)	
n.a.	2 (7)		0 (0)		2 (12)	
ENSAT Stage (%)		29/30		13/13		16/17
I	3 (10)		2 (15)		1 (6)	
II	12 (40)		3 (23)		9 (53)	
III	11 (37)		6 (46)		5 (29)	
IV	3 (10)		2 (15)		1 (6)	
n.a.	1 (3)		0 (0)		1 (6)	
Tumour size-diameter (cm)	9.7 ± 4.9	29/30	10.2 ± 4.9	13/13	9.3 ± 5.0	16/17
Total Weiss score	6 ± 2	30/30	6 ± 2	13/13	6 ± 1	17/17
Ki67 LI	18 ± 18	30/30	16 ± 11	13/13	20 ± 22	17/17
Resection status (%)		30/30		13/13		17/17
R0	18 (60)		3 (23)		15 (88)	
R1	6 (20)		5 (38)		1 (6)	
R2	1 (3)		1 (8)		0 (0)	
Rx	5 (17)		4 (31)		1 (6)	
MTT		28 (30)		13 (13)		15/17
Yes	27 (90)		13 (100)		14 (82)	
No	1 (3)		0 (0)		1 (6)	
n.a	2 (7)		0 (0)		2 (12)	
Chemotherapy (%)		26/30		11/13		15/17
No	17 (57)		7 (54)		10 (59)	
EDP	7 (23)		4 (31)		3 (18)	
Other	2 (7)		0 (0)		2 (12)	
n.a	4 (13)		2 (15)		2 (12)	
Progression (%)		29/30		13/13		16/17
Yes	11 (37)		5 (39)		6 (35)	
No	18 (60)		8 (61)		10 (59)	
n.a.	1 (3)		0 (0)		1 (6)	
Death (%)		29/30		13/13		16/17
Yes	6 (20)		4 (31)		2 (12)	
No	23 (77)		9 (69)		14 (82)	
n.a.	1 (3)		0 (0)		1 (6)	
Progression Free Survival Time (months)	38.0[20.5-82.0]	29/30	34[10-83.5]	13/13	38[21.5-88.2]	16/17
Overall Survival Time (months)	53.0[32.5-84.5]	29/30	62[21.5-83.5]	13/13	53[34.2-89.8]	16/17

Data are expressed as mean ± SD or median[IQR] according to their continuous normal or nonparametric distribution; categorical variables are expressed as number or percentage (%). NS: non-secreting; n.a.: not available; Ki67 LI: Ki67 Labelling Index; Tumor margin status is expressed as no residual tumor (R0), microscopic residual tumor (R1), macroscopic residual tumor (R2), uncertain resection status (Rx); MTT: Mitotane; EDP: etoposide, doxorubicin and cisplatin.

(IQR)]. Categorical variables were expressed as counts and percentages. Comparison between two groups of data was performed using Student's *t*-test for parametrically distributed variables and Mann-Whitney *U*'s test for nonparametric variables, while multiple comparisons were accomplished by one-way ANOVA test followed by post hoc Kruskal-Wallis' test for nonparametric variables. For the statistical analyses, the reference group is considered wild type (wt), consisting of those patients in the total cohort with ACC without any of the point mutations identified by the custom targeted-NGS panel. Overall survival (OS) has been defined as the probability that a patient diagnosed with the disease is still alive. Progression-free survival (PFS) has been defined as the probability of absence of any recurrence or metastasis or positive lymph nodes in stages < IV, or of any recurrence or increase in the number or diameter of distant lesions (including lymph nodes) in stage IV, according to the RECIST criteria[31] and ENSAT guidelines[5] by imaging (performing thorax plus complete abdomen Computed Tomography, every 3 months for the first 2 years and every 6 months till 5th year, and then annually till 10th year). Survival analysis was accomplished using the Kaplan-Meier method, and statistically significant differences between curves were estimated by the log-rank test. A P value <.05 was considered statistically significant and was also applied to the small samples deriving from subgrouping analysis. Hazard ratio of death from the tumour and disease progression was calculated with multivariable Cox regression, including point alterations in genes and their involved pathways, as well as age as independent discrete variables, considering wt or age <50 years to have risk = 1.

Results

Local cohort

The local cohort consisted of 30 conventional ACCs, with a gender ratio of 11 males to 19 females and an average age of 50 ± 11 years at diagnosis. The distribution of ENSAT stages was similar, with 50% classified as low-stage and 47% as high-stage. Over a follow-up period of 53.0 months (IQR:

Table 2. List of the driver genes identified in the literature as associated to ACC.

Gene	Cytogenetic location	Refseq transcript	Pathway affected	Alteration frequency in ACC	References
CDKN2A	9p21.3	NM_000077	p53 apoptosis/Rb1 cell cycle	11%-15%	16-18
CTNNB1	3p22.1	NM_001098210	Wnt/β-catenin signalling	15%-16%	16,25
DAXX	6p21.32	NM_001141969	Chromatin remodelling/maintenance	6%-7%	16,26
MED12	Xq13.1	NM_005120	Chromatin remodelling/maintenance	5%	16
NF1	17q11.2	NM_000267	Ras-cAMP/ERK MAP kinase cascade (Ras-ERK)	3%-5%	17,25
PRKAR1A	17q24.2	NM_001276289	cAMP/PKA signalling	8%	17
RB1	13q14.2	NM_000321	p53 apoptosis/Rb1 cell cycle	7%	16
TERT	5p15.33	NM_001193376	Chromatin remodelling/maintenance	6%-14%	16,17
TP53	17p13.1	NM_000546	p53 apoptosis/Rb1 cell cycle	16%-21%	16,17,25
ZNRF3	22q12.1	NM_001206998	Wnt/β-catenin signalling	1.9%-21%	16-18,25

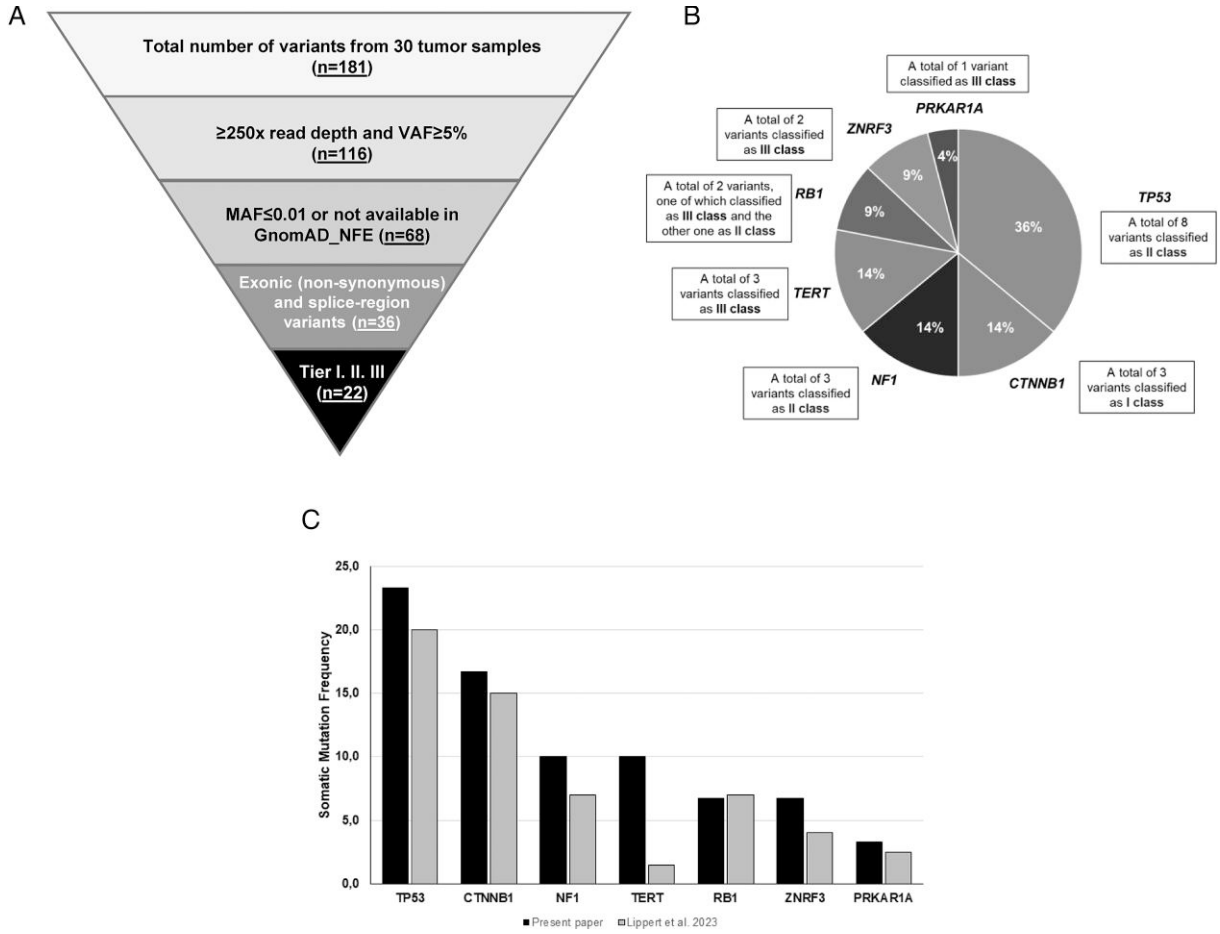

Figure 1. NGS genetic analysis of the local ACC cohort. Panel A: Inverted pyramid representation of bioinformatics filtering of the variants detected by targeted-NGS panel. MAF: Minor Allele Frequency in Non-Finnish Europeans based on GnomAD database; VAF: Variant Allele Frequency; Tier I: variants with strong clinical significance; Tier II: variants with potential clinical significance; Tier III: variants with unknown clinical significance. Panel B: Distribution of gene variants identified in the local ACC samples. Panel C: Mutation frequency of driver genes in the local ACC cohort of 30 patients (black columns) compared with the frequencies we calculated from the training cohort of 107 ACC patients (grey columns) in Lippert et al.

32.5-84.5), 11 patients experienced recurrence or tumour progression, and 6 died of the disease. Local series' characteristics are detailed in Table 1.

NGS analysis

Applying the custom NGS panel to the local cohort, for 9 out of the 10 ACC driver genes investigated we identified 181 variants that were filtered to a total of 116 on the basis of read depth and variant allele frequency (VAF). For clinical setting purposes, we started performing NGS on frozen tumour specimens to avoid any potential DNA degradation and fragmentation due to fixation. However, finding the same mutations in both frozen and FFPE tumour material in one patient, who carried mutations in 3 different candidate genes, confirmed that NGS can be applied to FFPE without losing any variants (not shown).[32] The overall variant filtering is illustrated in Figure 1A. Twenty-two rare variants were selected within coding regions and exon-intron boundaries including splicing, missense and nonsense variants, and classified of strong/

Table 3. Clinical characteristics of the 86 patients extracted from the ACC-TCGA cohort.

	ACC TCGA Cohort	N = 86	WT	N = 54	Patients with Mutation/ Variant	N = 32
Age (years)	47 ± 16	86/86	46 ± 17	54/54	49 ± 16	32/32
Male sex (%)	30 (35)	86/86	20 (37)	54/54	10 (31)	32/32
Secretion (%)		6/86		2/54		4/32
Cortisol	3 (4)		0 (0)		3 (9)	
Androgens	2 (2)		1 (2)		1 (3)	
Aldosterone	1 (1)		1 (2)		0 (0)	
NS	0 (0)		0 (0)		0 (0)	
n.a.	80 (93)		52 (96)		28 (88)	
Stage[a] (%)		84/86		53/54		31/32
I	9 (11)		6 (11)		3 (9)	
II	44 (51)		33 (61)		11 (34)	
III	11 (13)		7 (13)		4 (13)	
IV	20 (23)		7 (13)		13 (41)	
n.a.	2 (2)		1 (2)		1 (3)	
Tumor size diameter (cm)	10.8 ± 3.9	83/86	10.5 ± 4.0	53/54	11.1 ± 3.7	30/32
Total Weiss score	6 ± 2	47/86	5 ± 2	29/54	6 ± 2	18/32
KI67 LI	20 ± 18	27/86	13 ± 8	14/54	27 ± 23	13/32
Resection Status (%)		18/86		11/54		7/32
R0	15 (17)		11 (20)		4 (13)	
R1	3 (4)		0 (0)		3 (9)	
R2	0 (0)		0 (0)		0 (0)	
Rx	0 (0)		0 (0)		0 (0)	
n.a.	68 (79)		43 (80)		25 (78)	
Chemotherapy		80/86		51/54		29/32
Yes	58 (67)		33 (61)		25 (78)	
No	22 (26)		18 (33)		4 (13)	
n.a.	6 (7)		3 (6)		3 (9)	
Progression (%)		86/86		54/54		32/32
Yes	48 (56)		24 (44)		24 (75)	
No	38 (44)		30 (56)		8 (25)	
Death (%)		86/86		54/54		32/32
Yes	33 (38)		14 (26)		19 (59)	
No	53 (62)		40 (74)		13 (41)	
Progression Free Survival Time (months)	22.5[7.4-44.5]	85/86	31.2[14.1-61.1]	53/54	13.7[4.9-19.5]	32/32
Overall Survival Time (months)	38.5[19.6-66.4]	85/86	44.5[28.5-74.5]	53/54	22.0[15.5-45.5]	32/32

Data are expressed as mean ± SD or median[IQR] according to their continuous normal or nonparametric distribution; categorical variables are expressed as number or percentage (%). Stage is reported according to the American Joint Committee on Cancer Tumour Stage Code[a]. NS: non-secreting n.a.: not available; Ki67 LI: Ki67 Labelling Index; Tumour margin status is expressed as no residual tumour (R0), microscopic residual tumour (R1), macroscopic residual tumour (R2), uncertain resection status (Rx).

potential/unknown clinical significance (files) according to the literature.[33,34] To confirm the "somatic" nature of the tumour alterations (occurring during the life time in the tumour tissue) rather than the "germline" presentation (present in normal tissue), each variant was excluded by Sanger sequencing from the blood DNA extracted from leukocytes (not shown).

The majority of variants were missense ($n = 16$), $n = 5$ nonsense substitutions and 1 splicing variant (Table 2); 15/22 (68%) were classified as damaging and 7/22 (32%) of unknown significance (VUS, 31). All filtered variants in *CTNNB1*, *NF1* and *TP53* genes showed strong clinical significance (pathogenic), while those in *PRKAR1A*, *TERT* and *ZNRF3* genes ($n = 7$) were VUS. A *CTNNB1* variant (c.133T > C; p.Ser45Pro) was detected in more than one patient, confirming its role as a mutational hotspot. The final variants are all reported in the Catalogue of Somatic Mutations in Cancer (COSMIC), with the exception of the *NF1* splicing variant and the three *TERT* variants. *TP53* accounted for 36% ($n = 8/22$) of all alterations, encompassing 6 missense and 2 nonsense private variants, each classified as Tier II-with potential clinical significance (Figure 1B). *CTNNB1*, *NF1*, and *TERT* variants totalled 14% ($n = 3/22$).

Gene mutation frequency was 23% for *TP53*, followed by *CTNNB1* (17%), NF1 (10%) and *TERT* (10%) (Figure 1C). *RB1* and *ZNRF3* inactivating variants were detected in 7% of the cases, while the *PRKAR1A* gene was mutated in only 1 (3%) patient (Figure 1C). No interesting point mutations were identified in *CDKN2A* and *MED12* genes. Thus, 17 (57%) tumours harboured at least one variant affecting ACC-related pathways. The mutation frequency of the 7 genes in our cohort is consistent with the data reported by Lippert and colleagues,[29] with the exception of *TERT* (Figure 1C). Interestingly, mutational signature of the local cohort was characterised by a predominance of C > T, followed by T > C and C > A transitions (Table 4, Table S2).

In silico prediction of VUS

In silico analysis of the identified VUS (Table 4) revealed no impact of the three TERT mutations on the RNA-dependent DNA polymerase domains and on the location of the RNA-binding domain of the protein, with no alteration in the three-dimensional structure (Figure 2A). The p.Arg245Ter variant contributed to the deletion of the zinc-finger domains

Table 4. List of the selected gene variants found in the cohort ($n = 22$).

Gene	Transcript	cDNA	protein	Variant type	Tier	ACMG classification	Legacy ID (COSMIC)	Number of carriers
CTNNB1	NM_001098209	c.95A > G	p. Asp32Gly	missense	I	Pathogenic	COSM29417	1
CTNNB1	NM_001098209	c.133T > C	p.Ser45Pro	missense	I	Pathogenic	COSM5663	3
CTNNB1	NM_001098209	c.133T > G	p.Ser45Ala	missense	I	Pathogenic	COSM5685	1
NF1	NM_000267	c. 1885G > A	p.Gly629Arg	missense	II	Pathogenic	COSM220089	1
NF1	NM_000267	c.4270-1G > C		splicing	II	Pathogenic	n.a	1
NF1	NM_000267	c.574C > T	p.Arg192Ter	nonsense	II	Pathogenic	COSM42794	1
PRKAR1A	NM_001276289	c.797C > T	p.Thr266Met	missense	III	VUS	COSM5637227	1
RB1	NM_000321	c.2042G > A	p.Trp681Ter	nonsense	II	Pathogenic	COSM6908592	1
RB1	NM_000321	c.2650G > A	p.Glu884Lys	missense	III	VUS	COSM5786872	1
TERT	NM_001193376	c.430G > T	p.Val144Leu	missense	III	VUS	n.a	1
TERT	NM_001193376	c.237G > T	p. Glu79Asp	missense	III	VUS	n.a	1
TERT	NM_001193376	c.26C > T	p.Ala9Val	missense	III	VUS	n.a	1
TP53	NM_000546	c.584T > C	p. Ile195Thr	missense	II	Pathogenic	COSM329743	1
TP53	NM_000546	c.824G > T	p. Cys275Phe	missense	II	Pathogenic	COSM6022906	1
TP53	NM_000546	c.1024C > T	p.Arg342Ter	nonsense	II	Pathogenic	COSM220089	1
TP53	NM_000546	c.799C > T	p.Arg267Trp	missense	II	Pathogenic	COSM1169538	1
TP53	NM_000546	c.376T > G	p.Tyr126Asp	missense	II	Pathogenic	COSM6024609	1
TP53	NM_000546	c.541C > T	p.Arg181Cys	missense	II	Pathogenic	COSM11090	1
TP53	NM_000546	c.1031T > C	p.Leu344Pro	missense	II	Pathogenic	COSM44070	1
TP53	NM_000546	c.916C > T	p.Arg306Ter	nonsense	II	Pathogenic	COSM10663	1
ZNRF3	NM_001206998	c.992C > T	p.Prp331Leu	missense	III	VUS	COSM9726844	1
ZNRF3	NM_001206998	c.733C > T	p.Arg245Ter	nonsense	III	VUS	COSM1033089	1

For each variant, transcript, changes on cDNA and protein, variant type, Tier classification, ACMG classification, COSMIC Identifier (ID) and number of carriers are reported. n.a.: not available; VUS: Variant of Unknown Significance.

necessary for the negative regulation of the Wnt/β-catenin signalling pathway. The p.Pro331Leu ZNRF3 change modified the location of the zinc-finger domains in relation to the cytoplasmic cadherin domain, leading to a significant protein compression from 126.69 to 119.23Å (Figure 2B). The p.Thr266Met substitution in the PRKAR1A protein resulted in cyclic nucleotide-binding domain misfolding, changing the spatial distribution (Figure 2C). The p.Glu884Lys RB change altered the spatial distribution of the pocket domains causing protein stirring from 71.04 to 79.30 Å, preventing Rb1 transcriptional repressor activity on cell-cycle genes (Figure 2D). Therefore, the variants identified in *ZNRF3, PRKAR1A, RB1* genes were now be considered as likely pathogenic, while those in TERT likely benign.

Survival analysis

Somatic alterations and clinical characteristics for each patient of the local cohort are reported in Table 5 and Figure 3A. The mutated genes were clustered into main signalling pathways, the most frequently altered being Rb/p53 pathway (47%: *TP53*, *RB1*), followed by the Wnt/β-catenin pathway (41%: *CTNNB1*, *ZNRF3*), then *NF1 (*always present with *TP53* mutation, 18%) and *TERT* (always alone, 18%) genes. The only *PRKAR1A* variant was associated with *CTNNB1*.

The preliminary survival evaluation performed by Kaplan-Meier analysis of the local ACC cohort stratified in 4 classes according to the mutated signalling pathways or presentation in combined gene mutations, displayed a statistically significant difference in the velocity of tumour progression (pooled Log rank = .012). Patients with combined *NF1* + *TP53* alterations showed the most rapid progression (PFS = 20[3-20] months, $n = 3$), followed by patients with mutations in the Wnt/β-catenin (PFS = 38.0[24.5-47.0] months, $n = 5$) and Rb/p53 (PFS = 51.0[22.5-101.5] months, $n = 5$) pathways, while patients with *TERT* variants exhibited the slowest tumour progression (PFS = 99[68-99] months, $n = 3$), being these differences between the median PFS statistically significant ($P = .038$, Kruskal-Wallis' test). The statistical significance was retained even when Kaplan-Meier analysis was limited to the low-stage ACCs (I-II) with a log rank = .014. Differences in OS did not reach any statistical significance (pooled Log rank = .147), though mortality in patients with altered Wnt/β-catenin pathway was 100% by month 74.

To increase the power of the survival analysis, we extended the local cohort including data from patients of the ACC-TCGA cohort[17] with conventional tumours bearing point mutations in the same genes we identified with the targeted-NGS panel. The new cohort consisted of 113 patients, with $n = 46$ (41%) bearing at least one pathogenic/likely pathogenic point mutation in the selected genes and their combinations, and $n = 67$ without any of the above point mutations (wild type: wt). ACC-TCGA characteristics are reported in Table 3 and Figure 3B. An additional mutation class was present only in the ACC-TCGA, consisting of combined single mutations in Rb/p53 + Wnt/β-catenin pathways.

Kaplan-Meier analysis of the total cohort confirmed the preliminary results of the local cohort highlighting differences in the behaviour of the survival curves (Figure 4A-C for PFS and Figure 4G-I for OS). For PFS, statistically significant differences in tumour progression were present vs wt for *NF1* + *TP53* combination (pairwise log rank = .039) and for Rb/p53 + Wnt/β-catenin pathway (pairwise log rank < .001), Figure 4A, which were associated to a significant reduction in PFS vs wt ($P = .009$ and $P = .005$, respectively), Figure 4D. A similar survival behaviour was appreciated when patients were grouped in low-(I/II) vs high-(III-IV) stages (Figure 4B vs 4C); the statistical significance in PFS time reduction was maintained only in the low (Figure 4E) but not in the high stages (Figure 4F), except for Wnt/β-catenin pathway. Any mutation in Wnt/β-catenin + Rb/p53 pathways conferred the worst progression behaviour (Figure 4A, B, D, E), also compared with mutations in the

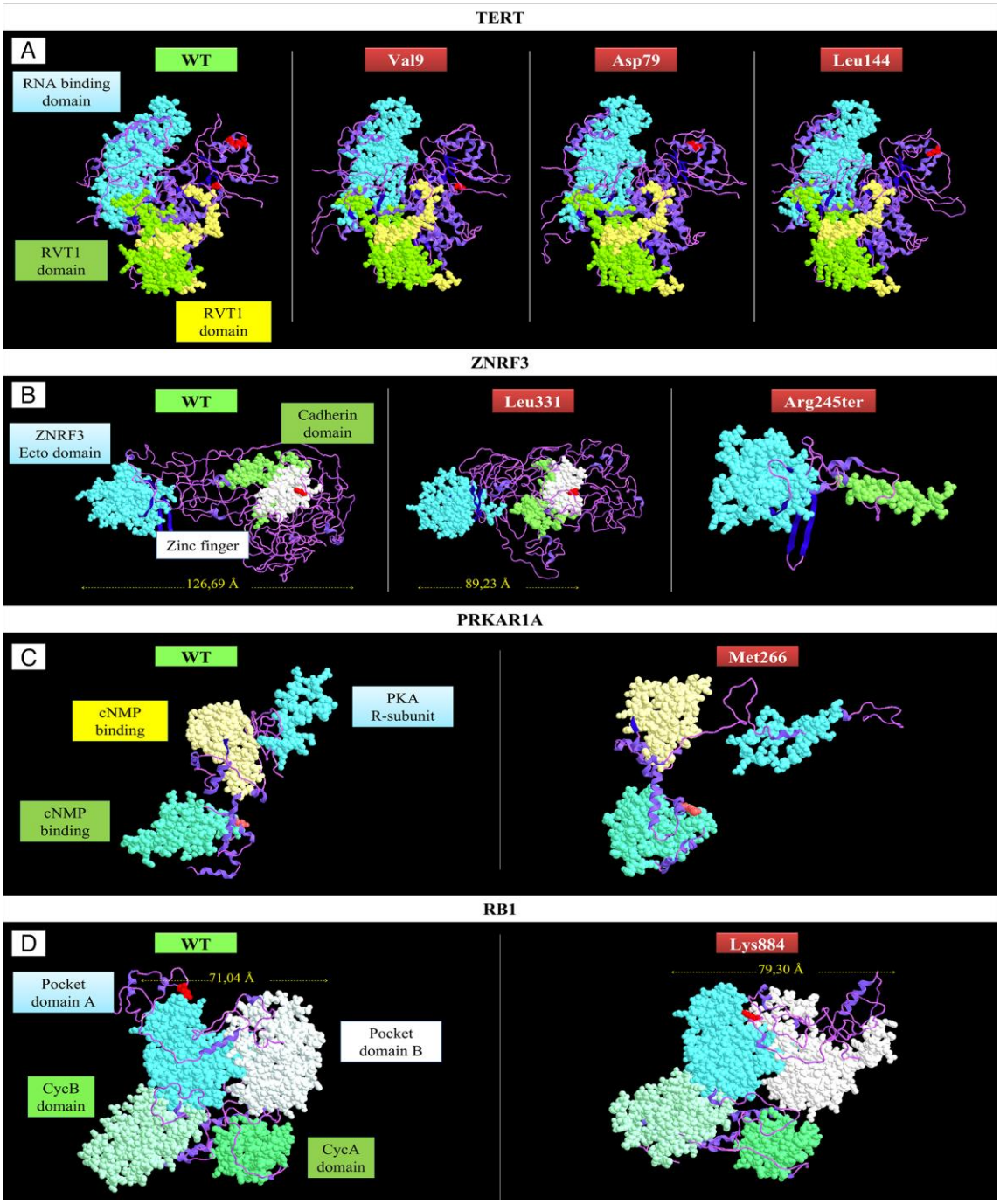

Figure 2. *In silico* prediction of the tertiary structures of the protein encoded by mutated genes. Phyre2 software (Protein Homologyfold Recognition Server; www.sbg.bio.ic.ac.uk/phyre2/) was used to assess potential functional effects of the variants seen in *TERT* (panel A), *ZNRF3* (panel B), *PRKAR1A* (panel C) and *RB1* (panel D) genes. The amino acid variants associated with each mutation are indicated, and predicted 3D structures of mutated proteins are compared with the predicted conformation of each wt encoding gene. Specific functional domains are indicated with different colours.

single pathway ($P = .029$ vs Rb/p53 Figure 4D and $P = .027$ vs Wnt/β-catenin pathways, Figure 4E). For OS curves, when considering all stages, Rb/p53 + Wnt/β-catenin and Wnt/β-catenin mutated pathways had the worst behaviour (Figure 4G), which was however statistically significant for OS median time (Figure 4J) only for the two combinations *NF1* + *TP53* ($P = .025$) and Rb/p53 +Wnt/β-catenin pathways ($P = .024$), Figure 4J. This statistical significance was also maintained for the two combinations in low-(Figure 4K) but not in high-(Figure 4L) stages. Interestingly, while the combined mutation in Rb/p53 + Wnt/β-catenin pathways had the worst prognosis for OS and PFS and in both low and high-stage patients, PFS seemed to be more affected by the combination *NF1* + *TP53* in low-stage patients (Figure 4B), while the Wnt/β-catenin pathway was crucial in high stage patients (Figure 4C).

A multivariable survival-risk analysis associated with point mutations in the different signalling pathways compared with

Table 5. Genotype and clinical features of each ACC patient in the cohort.

Case	Sex	Age (ys)	Secretion	ENSAT stage	size (cm)	Ki67 (%)	Weiss	Genotype	VAF (%)	AMP	ACMG
3	M	64	C	II	7,5	30	7	**CTNNB1** (NM_001098209):c.95A > G; p.Asp32Gly	32,8	I	Pathogenic
5	F	62	NS	II	2,5	10	6	**TERT** (NM_001193376):c.430G > T; p.Val144Leu	27,65	III	VUS
6	M	26	C + ANDRO	II	14,5	15	7	Non relevant variants			
7	F	59	NS	II	7,5	30	8	Non relevant variants			
8	M	47	C	III	18,0	15	8	Non relevant variants			
9	F	51	ALDO	III	3,0	20	5	**TP53** (NM_000546):c.584T > C; p.Ile195Thr	22,62	II	Pathogenic
10	F	58	ANDRO	III	9,5	30	7	**TERT** (NM_001193376):c.237G > T; p.Glu79Asp	19,72	III	VUS
11	F	58	NS	III	13,0	90	8	**TP53** (NM_000546):c.824G > T; p.Cys275Phe	17,18	II	Pathogenic
13	F	58	C	III	7,0	40	3	Non relevant variants			
16	F	45	ANDRO	II	7,5	5	6	Non relevant variants			
17	F	37	n.a.	II	n.a.	5	6	**TERT** (NM_001193376):c.26C > T; p.Ala9Val	13,12	III	VUS
21	M	61	C	I	3,0	5	3	**CTNNB1** (NM_001098209):c.133T > C; p.Ser45Pro	26,9	I	Pathogenic
22	F	71	NS	IV	9,0	10	7	**CTNNB1** (NM_001098209):c.133T > G; p.Ser45Ala	68,21	I	Pathogenic
23	F	46	C	n.a.	7,5	1	4	**CTNNB1** (NM_001098209):c.133T > C; p.Ser45Pro	34,85	I	Pathogenic
24	F	36	C	III	6,7	15	5	Non relevant variants			
25	M	54	NS	I	4,5	10	5	Non relevant variants			
26	F	27	n.a.	II	9,5	5	6	**ZNRF3** (NM_001206998):c.992C > T; p.Pro331Leu	67,93	III/IV	VUS
27	M	56	C	IV	15,0	30	8	Non relevant variants			
28	M	51	NS	IV	17,0	15	8	Non relevant variants			
29	**F**	**62**	ANDRO	III	4,8	25	8	**NF1** (NM_000267):c.1885G > A; p.Gly629Arg	90,5	II	Pathogenic
								TP53 (NM_000546):c.916C > T; p.Arg306Ter	85,25	II	Pathogenic
								ZNRF3 (NM_001206998):c.733C > T; p.Arg245Ter	81,17	III/IV	VUS
30	**F**	**54**	C	III	13,5	50	8	**RB1** (NM_000321):c.2042G > A; p.Asp681Ter	50	II	Pathogenic
								TP53 (NM_000546):c.799C > T; p.Arg267Trp	9,96	II	Pathogenic
								TP53 (NM_000546):c.1024C > T; p.Arg342Ter	44,61	II	Pathogenic
31	M	46	C	III	15,0	15	6	Non relevant variants			
32	F	56	NS	III	9,7	10	6	Non relevant variants			
33	M	52	ANDRO	I	4,6	2	4	Non relevant variants			
34	M	38	C	II	13,0	20	8	**CTNNB1** (NM_001098209):c.133T > C; p.Ser45Pro	39,38	I	Pathogenic
								PRKAR1A (NM_001276289):c.797C > T; p.Thr266Met	18,14	III	VUS
35	F	41	ANDRO	II	10,0	6	5	**RB1** (NM_000321):c.2650G > A; p.Glu884Lys	5,1	III	VUS
36	F	48	ANDRO	II	18,0	**5**	6	**NF1** (NM_000267):c.574C > T; p.Arg192Ter	40,89	II	Pathogenic
								TP53 (NM_000546):c.541C > T; p.Arg181Cys	13,3	II	Pathogenic
37	F	40	NS	II	6,5	**10**	6	**NF1** (NM_000267):c.4270-1G > C	70,85	II	Pathogenic
								TP53 (NM_000546):c.376T > G; p.Tyr126Asp	72,35	II	Pathogenic
38	F	66	C	III	5,5	10	6	Non relevant variants			
39	M	37	n.a.	II	6,5	10	n.a.	Non relevant variants			
40	F	52	NS	II	19	20	5	**TP53** (NM_000546):c.1031T > C; p.Leu344Pro	80,45	II	Pathogenic

C: cortisol; ALDO: aldosterone; ANDRO: androgens; NS: non secreting; n.a.: not available; the genes interested by the variant are indicated in bold.

the wt condition and adjusted for age as a dummy variable (<50 or ≥50 years) was run, Table 6. Among the different mutations, *NF1* + *TP53* combination and Rb/p53 + Wnt/β-catenin pathways displayed a statistically significant increased risk of progression compared with wt, which further increased when considering the low-stages. An even higher risk of death was associated to these two mutational groups in low stage patients. Conversely, in the high-stage, the prognostication relevance of these mutations was lost. Age effect has a different trend in low- vs high-stages.

Finally, the relevance of the number of point mutations for the survival outcomes (PFS and OS) was assessed with Kaplan-Meier (Figure S1) and multivariable Cox regression (Table S1). The presence of more than one pathogenic alteration in any genes was associated to significantly reduced PFS and OS time, even compared with having a single mutation (Figure S1) and increased HR (Table S1), but only when considering the low stages. Young age was confirmed also in this multivariable analysis to behave as a protective or detrimental factor in low or high-stages, respectively (Figure S1).

Discussion

Targeted-NGS has revolutionised medical genetic research by cutting sequencing costs while increasing the throughput, allowing simultaneous analysis of several genes with decreased allele dropout. Thus, multi-gene panels are becoming the standard approach for the molecular analysis of solid tumours.[29,35] Recently, a large multicentre study on 194 ACC samples showed that DNA-based biomarkers, evaluated by targeted sequencing of 160 cancer-specific genes for the training cohort as well as two smaller panels including 100 and 33 genes for the validation cohort, can improve prognostication beyond routinely available clinical and histopathological parameters.[29]

Here, starting from a local series of 30 ACCs, we demonstrated the clinical utility of tumour mutational analysis using a light custom targeted-NGS panel. Moreover, by incorporating data from the ACC-TCGA series, we compared the prognostic power of the different signalling pathways/combinations involving those genes harbouring any point mutations. In the local series, 116 variants were identified, filtered according to gene location, and classified as somatic variants of strong/potential/

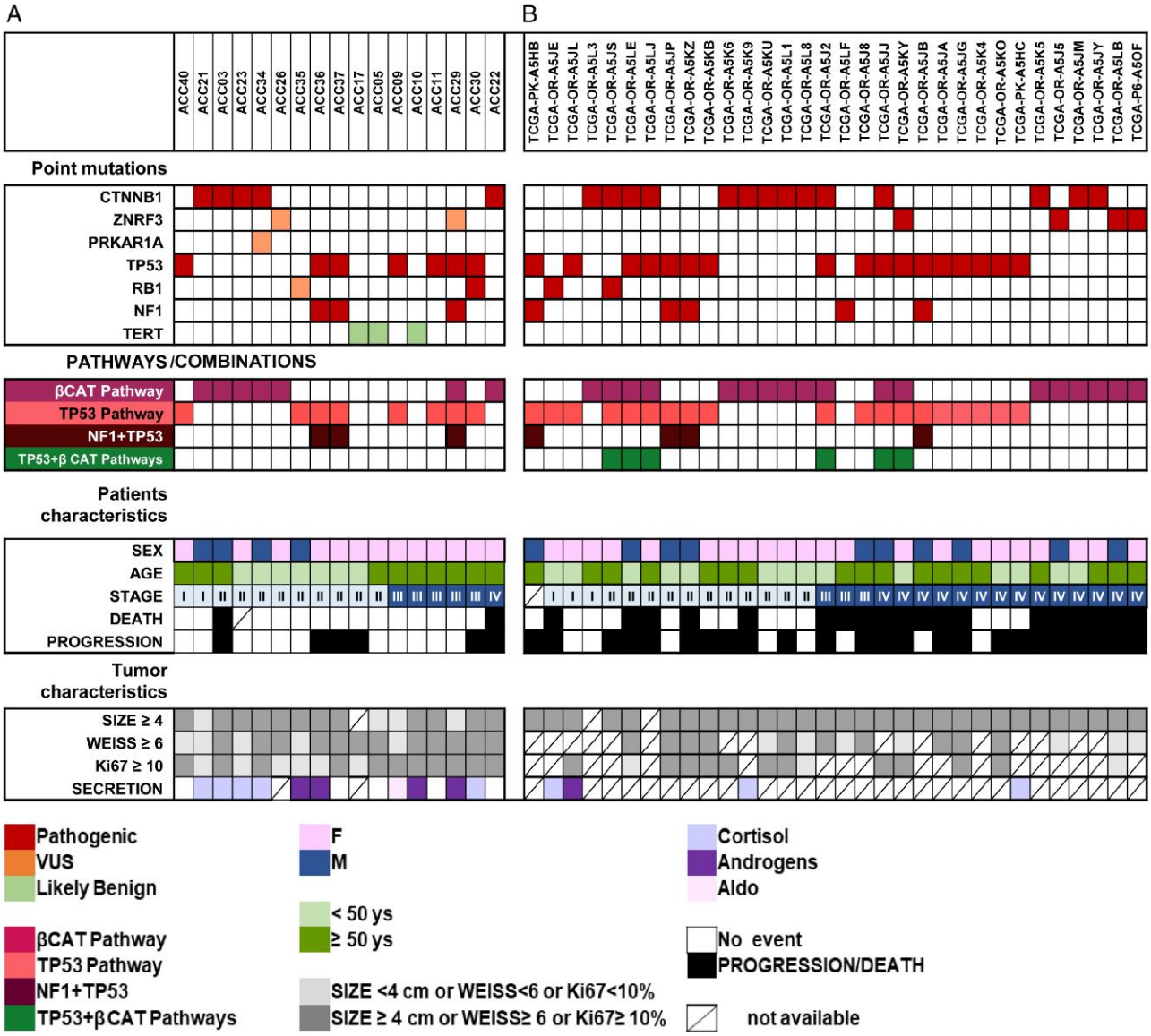

Figure 3. NGS point mutation signature and clinical profiles of mutated ACC from the local and ACC-TCGA cohorts. Heatmap describing distribution of the cancer-specific somatic point mutations found in each gene clustered according to the specific signalling pathways or combinations (upper panels) and the main clinical-pathological characteristics (lower panels) in the local cohort of $n = 17$ mutated patients (panel A) and in the $n = 32$ mutated patients from ACC-TCGA series (panel B). The colour legend is given below the heatmap. Cases are ordered by increasing tumour stages.

unknown clinical significance.[35] This stringent filtering limited the otherwise very high mutational burden previously found with a targeted approach,[36] and 22 variants in 7 genes (*CTNNB1, NF1, PRKAR1A, RB1, TERT, TP53* and *ZNRF3*) were obtained. Notably, 10% of our cases showed an allele frequency ≥70%. Given that germline conditions were excluded by blood analysis, the far higher VAFs could implicate loss-of-heterozygosity mechanisms in the cancer development. As previously reported, no point mutation was detected in the *CDKN2A* gene.[16,17] In line with the very low mutation frequency (1%) described by Lippert and colleagues, no point mutation for the *MED12* gene was found in our cohort.[29] The *DAXX* gene was removed from our analysis for insufficient sequence coverage (<50x). *TP53* was the most frequently mutated gene (23%), with a frequency higher than in other studies,[16,17] but similar to the 20% we calculated from Lippert's data.[29] Eight pathogenic *TP53* variants were identified, 6 missense and 2 nonsense, the majority affecting the DNA-binding domain, in agreement with the literature.[16,37] *CTNNB1* was the second most frequently mutated gene (16%).[16,17] All mutations were missense and confined to exon-3, which encodes the regulatory N-terminal amino acids.[38] Four of the five *CTNNB1*-mutated tumours harboured a codon 45 substitution, and the p.Ser45Pro hotspot was the most common *CTNNB1* somatic alteration, seen in three cases. *NF1* and *TERT* alterations were observed in 10% of the cases, in accordance with Ross and colleagues.[26] Mutations in the protein coding sequence of *TERT* gene have been assessed only recently, occurring in approximately 3% of cases.[29] We identified 3 missense VUS not affecting TERT protein function according to the *in silico* modelling performed. *RB1* and *ZNRF3* point alteration frequency was 6%, in line with the literature.[18,29] We found only one missense *PRKAR1A* variant with unknown significance (3%) as recently described.[29] In silico modelling of the novel VUS found, enabled us to better classify them as likely pathogenic and likely benign (Figure 3).

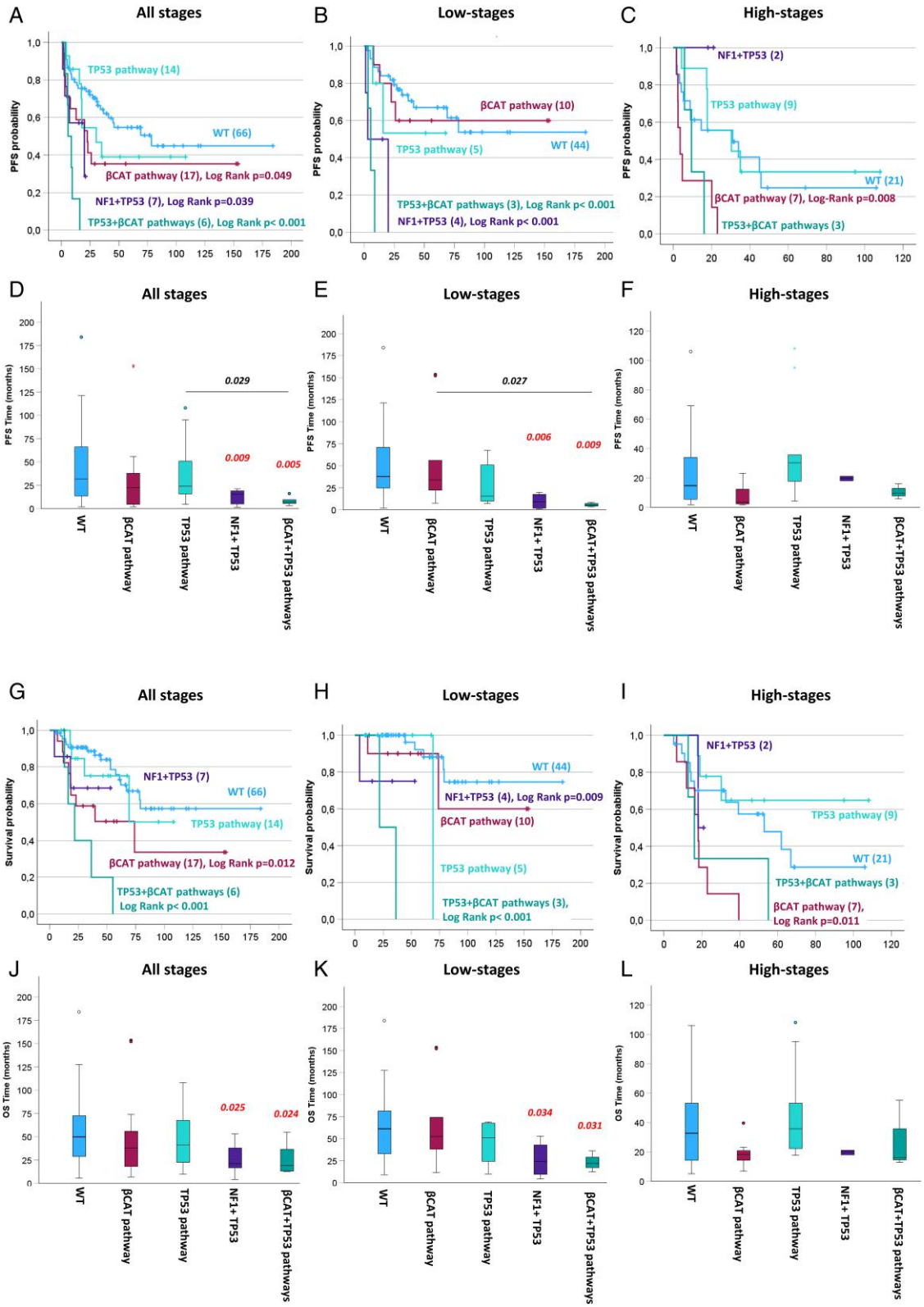

Figure 4. Kaplan-Meier of survival analysis in the total cohort of ACCpatients. Patients were stratified into five groups according to the pathways involving the mutated genes or the combination of the mutated genes or pathways. WT: nonmutated; βCAT pathway: Wnt/β-catenin pathway; TP53 pathway: Rb/p53 pathway; TP53+βCAT: combination of Rb/p53 + Wnt/β-catenin pathways; NF1 + TP53: combination of mutations in *NF1* and *TP53* genes. Panels A-C and G-I: Kaplan-Meier survival curves for the Progression Free Survival (PFS) and Overall Survival (OS) were calculated for all tumor stages (panels A, G: pooled Log Rank $P < .001$ for PFS & pooled Log Rank $P < .001$ for OS, respectively), low-stage (panels B, H: pooled Log Rank $P < .001$ for PFS & pooled Log Rank $P < .001$ for OS, respectively) and high-stage (panels C, I: pooled Log Rank $P = .008$ for PFS & pooled Log Rank $P = .018$ for OS, respectively) patients. The number of patients in each group is indicated in brackets, along with the statistically significant pairwise Log Rank values for each curve vs the WT curve. Panels D-F & J-L: Box charts indicate the median value of PFS (panels D-F) and OS (panels J-L) time in each group. Statistical significance was evaluated among groups by One-way ANOVA followed by *post-hoc* Kruskal-Wallis' test towards WT or as indicated, for all stages (panels D, J), low-stage (panels E, K) and high-stage (panels F, L) patients; $P < .05$ is considered statistically significant and indicated in bold Italics for comparison vs WT and underlined for comparison between the mutated genes and/or pathways.

Table 6. Multivariable survival analysis including the mutated gene pathways/combinations and age grouping in the total cohort of ACC patients.

	PROGRESSION			DEATH		
	Total cohort HR [95%CI]	**Low-stages HR [95%CI]**	**High-stages HR [95%CI]**	**Total cohort HR [95%CI]**	**Low-stages HR [95%CI]**	**High-stages HR [95%CI]**
Wnt/β catenin	HR = 1.97 [.98-3.98] P = .058	HR = 1.16 [.38-3.52] P = .788	HR = 6.24 [1.98-19.67] P = .002	HR = 2.413 [1.06-5.48] P = .035	HR = 1.23 [.22-6.80] P = .810	HR = 3.24 [1.12-9.41] P = .031
Rb/p53	HR = 1.38 [.62-3.06] P = .429	HR = 2.25 [.49-10.30] P = .296	HR = 1.24 [.42-3.65] P = .694	HR = 1.10 [.36-3.30] P = .870	HR = 8.00 [.74-86.39] P = .087	HR = .49 [.13-1.84] P = .288
NF1+ TP53	HR = 2.96 [1.01-8.69] P = .048	HR = 13.23 [3.15-55.61] P < .001	—	HR = 2.37 [.53-10.54] P = .259	HR = 54.81 [3.33-902.61] P = .005	HR = 1.38 [.16-11.89] P = .770
Rb/p53+ Wnt/β catenin	HR = 6.47 [2.54-16.49] P < .001	HR = 16.24 [3.87-68.00] P < .001	HR = 2.73 [.72-10.36] P = .141	HR = 7.44 [2.64-21.01] P < .001	HR = 39.11 [4.10-373.21] P = .001	HR = 2.84 [.76-10.67] P = .121
AGE	HR = 1.19 [.67-2.03] P = .521	HR = 2.42 [1.03-5.64] P = .042	HR = .41 [.16-1.01] P = .053	HR = 1.94 [.98-3.81] P = .055	HR = 5.97 [1.26-28.36] P = .025	HR = 1.28 [.51-3.21] P = .598

The hazard ratio (HR) of tumour progression and death was calculated for each mutated signalling pathways/combinations through a multivariable Cox regression compared with those patients bearing no point mutations in the analysed genes (wt). Age was considered as a discrete covariate (senior vs young age, with a cut off = 50 years). Risk analysis was considered for patients of all stages, as well as stratified in low- and high-stages. HR: hazard ratio; CI: confidence interval; (—) indicates the incapability of performing statistical analysis for the subgroup. Statistical significance corresponding to $P < .05$ is indicated in bold Italics.

Among the signalling pathways affected by point mutations, the most frequently altered in the local cohort was the Rb/p53 cell cycle (*TP53*, *RB1*, 47%), followed by Wnt/β-catenin (*CTNNB1, ZNRF3*, 41%) and Ras/MAPK (*NF1*, 18%) signalling. We showed here that *CTNNB1* and *ZNRF3* mutations were mutually exclusive and *TP53* can associate with *RB1* or *NF1* variants. Interestingly, by including the ACC-TCGA, an associated mutation in the Rb/p53 + Wnt/β-catenin pathways emerged, as previously described.[17,29,39,40] Finally, tumours with *NF1* impairment always harboured a *TP53* mutation in both ACC series, implying an association between these two genes mapping on ch17. The high frequency of somatic *NF1* mutations in sporadic tumours indicates that neurofibromin may play a role in cancer far beyond the predisposition evident in NF1 tumour syndrome.[41] *NF1* + *TP53* associated mutation deserves further investigations. Notably, in the local cohort, we observed a mutational signature of the retained somatic variants, characterised by a predominance of C > T, followed by T > C and C > A transitions. Suggestively, the most represented single-base substitution mutational signatures (SBS) concerning C > T transitions was SBS1 (C > T at NCG nucleotides) demonstrated as associated with age and found in all types of cancer.[42,43] SBS4, characterised by C > A transition, directly associated to tobacco smoke induced DNA mutagenesis, has previously been described in ACC.[17,44]

In the total cohort of 113 ACC patients, we were able to perform a solid survival analysis. To the best of our knowledge, this is the first time that a direct comparison of the prognostic ability of point mutations in different signalling pathways has been undertaken. In the ACC-TCGA, 3 clusters of genes derived from integration of a multi-genomic analysis were identified displaying different prognostication power and associated with different mutational pathways.[17,45] However, that analysis was performed using an untargeted multi-genomic complex approach, whereas, here, we propose a light custom targeted-NGS panel easily transferrable to routine analysis.

By Kaplan-Meier univariate survival analysis, we showed a different relative velocity of tumour progression and death, according to the point mutational pathways identified: the combined alterations in *TP53* + *NF1* genes, and even to a higher extent, in the Wnt/β-catenin + Rb/p53 pathways, displayed a significantly reduced time to progression and OS time, likely cooperating in exacerbating the malignant trait of the single mutations, as previously demonstrated for the latter combined pathways.[16,17,29,40] This is coherent with the findings observed in ACC-genetic mouse models where the concurrent Wnt/β-catenin mutational activation cooperates with the loss of p53 to promote murine ACC tumourigenesis and progression.[46,47] The importance of identifying point mutational pathways in ACC patients, independently from the tumour stage, was further confirmed by the observed increase in HR for OS and PFS associated to all the pathways bearing pathogenic gene alterations compared with wt. HRs associated to combined mutations in the *TP53* + *NF1* genes and Wnt/β-catenin + Rb/p53 pathways were statistically significant and even higher when calculated in the low-stages (I, II), while no significance was evident in the high-stage groups in a multivariable Cox regression analysis adjusted for age. Of note, the Wnt/β-catenin pathways appeared to have a significantly increased HR for OS outcome in advanced stages (III-IV). These findings suggest that in low-stages, point mutations in these genes play a relevant role for progression and OS, while in advanced stages, probably other risk factors are more

important, except for mutations in the Wnt/β-catenin pathways which, even when alone, is associated to 100% of death.

Alterations in the Wnt/β-catenin pathway were associated with death in all the mutated cases, while there were no deaths in those harbouring mutations in other pathways. Consequently, patients with Wnt/β-catenin mutations should receive a stringent follow-up and aggressive treatment, independently of the tumour stage. Of note, the multi-genomic CoC analysis identified alterations in Wnt/β-catenin and cell cycle pathways, as mainly associated with the most aggressive COC3 (Cluster-of-Cluster 3), characterised by the worst prognosis.[17,45]

Similar to previous studies,[29,40] we also confirmed the relevance of the number of point mutations, associated to a statistically significant increase in HR for both death and progression, in our analysis, when limited to the low-stages.

Interestingly, in all multivariable Cox regression analyses, aging appeared detrimental or protective in low- or in high-stages, respectively.

Our study recognizes some limitations: (1) the panel designed lacks some driver genes and should be improved; (2) NGS analysis was performed on DNA extracted from frozen samples and not from FFPE; (3) the synonymous variants are excluded from our analysis, although some may have functional effects[48,49]; (4) our genetic analysis targeted point mutations without encompassing CNV and DNA methylation; (5) we cannot exclude that the combination of the two datasets (local and TCGA cohort) have introduced potential biases; (6) due to ACC rarity, the number of patients in the monocentric local cohort screened by the custom targeted-NGS panel is low and could affect the results. However, the inclusion of data from the ACC-TCGA allowed a solid survival analysis, though some information (Ki67-LI, R status) were not extractable for the majority of those patients; (7) the small sample size introduced by sub-grouping could result in a bias in the statistical analysis; (8) the study is retrospective and even benefiting from the TCGA, the sample size can still be increased, therefore validation in a larger prospective study and on routine FFPE tumour material is mandatory to confirm our preliminary findings.

Conclusions

Based on our findings, parallel sequencing of multiple targeted driver genes appears to be the first step towards routine genetic characterisation of surgically treated ACC for prognostic purposes. Targeted-NGS analysis may improve the clinical management of low-risk patients by identifying the need for more stringent surveillance and personalised treatment.

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Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Authors' contributions

Francesca Cioppi (Conceptualisation [equal], Investigation [equal], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Giulia Cantini (Conceptualisation [equal], Data curation [equal], Investigation [equal], Methodology [equal], Supervision [equal], Writing—original draft [equal], Writing—review & editing [equal]), Tonino Ercolino (Formal analysis [equal], Investigation [equal], Methodology [equal], Writing—review & editing [equal]), Massimiliano Chetta (Investigation [equal], Methodology [equal], Software [equal], Writing—review & editing [equal]), Lorenzo Zanatta (Formal analysis [equal], Writing—review & editing [equal]), Gabriella Nesi (Investigation [equal], Writing—review & editing [equal]), Massimo Mannelli (Conceptualisation [equal], Writing—review & editing [equal]), Mario Maggi (Writing—review & editing [equal]), Letizia Canu (Conceptualisation [equal], Data curation [equal], Writing—review & editing [equal]), and Michaela Luconi (Conceptualisation [equal], Funding acquisition [equal], Supervision [equal], Writing—original draft [equal], Writing—review & editing [equal])

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