Assessment of VAV2 Expression Refines Prognostic Prediction in Adrenocortical Carcinoma

Silviu Sbiera,[1] Iuliu Sbiera,[1] Carmen Ruggiero,[2,3,4,5] Mabrouka Doghman-Bouguerra,[2,3,4,5] Esther Korpershoek,[6] Ronald R. de Krijger,[6,7] Hester Ettaieb,[8] Harm Haak,[8,9,10] Marco Volante,[11] Mauro Papotti,[11] Giuseppe Reimondo,[12] Massimo Terzolo,[12] Michaela Luconi,[13] Gabriella Nesi,[13] Massimo Mannelli,[13] Rossella Libé,[14,15,16] Bruno Ragazzon,[14,15,16] Guillaume Assié,[14,15,16] Jérôme Bertherat,[14,15,16] Barbara Altieri,[1,17] Guido Fadda,[18] Natalie Rogowski-Lehmann,[19] Martin Reincke,[19] Felix Beuschlein,[19,20] Martin Fassnacht,[21] and Enzo Lalli[2,3,4,5]

[1]Department of Internal Medicine I – Division of Endocrinology and Diabetes, University Hospital, University of Würzburg, 97080 Wurzburg, Germany; [2]Université Côte d'Azur, Sophia Antipolis, 06560 Valbonne, France; [3]CNRS UMR7275, Sophia Antipolis, 06560 Valbonne, France; [4]NEOGENEX CNRS International Associated Laboratory, Sophia Antipolis, 06560 Valbonne, France; [5]Institut de Pharmacologie Moléculaire et Cellulaire, Sophia Antipolis, 06560 Valbonne, France; [6]Department of Pathology, Erasmus MC Cancer Institute, University Medical Center, 3000 CA Rotterdam, The Netherlands; [7]Department of Pathology, Reinier de Graaf Hospital, 2625 AD Delft, The Netherlands; [8]Department of Internal Medicine, Máxima Medical Centre, 5631 BM Eindhoven/Veldhoven, The Netherlands; [9]Department of Internal Medicine, Division of General Internal Medicine, Maastricht University Medical Centre+, 6202 AZ Maastricht, The Netherlands; [10]Maastricht University, CAPHRI School for Public Health and Primary Care, Ageing and Long-Term Care, 6200 MD Maastricht, The Netherlands; [11]Department of Oncology, University of Turin at San Luigi Hospital, 10043 Orbassano, Italy; [12]Department of Clinical and Biological Sciences, University of Turin at San Luigi Hospital, 10043 Orbassano, Italy; [13]Department of Experimental and Clinical Biomedical Sciences "Mario Serio," University of Florence, 50139 Florence, Italy; [14]Inserm U1016, Institut Cochin, 75014 Paris, France; [15]CNRS UMR8104, 75014 Paris, France; [16]Université Paris Descartes, Sorbonne Paris Cité, 75014 Paris, France; [17]Division of Endocrinology and Metabolic Diseases, Catholic University of the Sacred Heart, 00168 Rome, Italy; [18]Division of Anatomic Pathology and Histology, Catholic University of the Sacred Heart, 00168 Rome, Italy; [19]Medizinische Klinik and Poliklinik IV, Ludwig-Maximilians-Universität, 80336 Munich, Germany; [20]Klinik für Endokrinologie, Diabetologie und Klinische Ernährung, Universitätsspital Zürich, 8091 Zurich, Switzerland; and [21]Comprehensive Cancer Center Mainfranken, University of Würzburg, 97080 Wurzburg, Germany

Context: Adrenocortical carcinoma (ACC) is a rare endocrine malignancy with overall poor prognosis. The Ki67 labeling index (LI) has a major prognostic role in localized ACC after complete resection, but its estimates may suffer from considerable intra- and interobserver variability. VAV2 overexpression induced by increased Steroidogenic Factor-1 dosage is an essential factor driving ACC tumor cell invasion.

Objective: To assess the prognostic role of VAV2 expression in ACC by investigation of a large cohort of patients.

Design, Setting, and Participants: A total of 171 ACC cases (157 primary tumors, six local recurrences, eight metastases) from seven European Network for the Study of Adrenal Tumors centers were studied.

Outcome Measurements: H-scores were generated to quantify VAV2 expression. VAV2 expression was divided into two categories: low (H-score, <2) and high (H-score, ≥ 2). The Ki67 LI retrieved from patients' pathology records was also categorized into low ($<20\%$) and high ($\geq 20\%$). Clinical and

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received 26 April 2017. Accepted 28 June 2017.
First Published Online 3 July 2017

Abbreviations: ACC, adrenocortical carcinoma; CI, confidence interval; ENS@T, European Network for the Study of Adrenal Tumors; HR, hazard ratio; LI, labeling index; OS, overall survival; PFS, progression-free survival; SD, standard deviation; SF-1, Steroidogenic Factor-1; TMA, tissue microarray.

immunohistochemical markers were correlated with progression-free survival (PFS) and overall survival (OS).

Results: VAV2 expression and Ki67 LI were significantly correlated with each other and with PFS and OS. Heterogeneity of VAV2 expression inside the same tumor was very low. Combined assessment of VAV2 expression and Ki67 LI improved patient stratification to low-risk and high-risk groups.

Conclusion: Combined assessment of Ki67 LI and VAV2 expression improves prognostic prediction in ACC. (***J Clin Endocrinol Metab*** **102: 3491–3498, 2017)**

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy with overall poor prognosis, limited treatment options when progressed into metastatic stage, and unsatisfactory response to polychemotherapeutic cytotoxic regimens (1, 2). Hence the most efficient method to eradicate the disease consists in complete surgical resection of the primary tumor. However, risk of recurrence is high even in this condition. Molecular studies have identified two subclasses of ACCs with aggressive or indolent clinical behavior (3–6). However, because molecular markers identified by those studies have not yet found entrance into clinical practice, it would be of particular importance to stratify patients with ACC into low- or high-risk groups to adequately monitor disease recurrence and assign them to appropriate therapeutic interventions. The histological Weiss score, which is commonly used as an established morphometric criterion for differential diagnosis in adrenocortical tumors, has limited value as a prognostic indicator, especially in cases with borderline features (7, 8). Conversely, it was shown that several immunohistochemical markers have a prognostic value in ACC (9–18). Among those, the most widely used in clinical pathology reports is the Ki67 labeling index (LI), which is directly related to the proliferative activity of a given tissue (14–18). A study recently completed by the European Network for the Study of Adrenal Tumors (ENS@T) could indeed demonstrate that Ki67 LI has a major prognostic role in localized ACC after complete resection (18). However, Ki67 LI estimates suffer from considerable intra- and interobserver variability, as highlighted in a recent study (19). New prognostic markers are needed, therefore, to further refine prognostic classification of patients with ACC as part of a multiparametric analysis.

The transcription factor Steroidogenic Factor-1 (SF-1) has a pivotal role in regulating adrenocortical cell proliferation and differentiation (20). Its overexpression is associated with adrenocortical tumorigenesis through regulation of a specific set of SF-1 dosage-dependent target genes (21, 22). One of these genes encodes VAV2, a guanine nucleotide exchange factor for small GTPases of the Rho family (23). We have recently shown that VAV2 overexpression induced by an increased SF-1 dosage in ACC is an essential factor driving tumor cell invasion (24). Herein, we present the results of a large study involving ACC cases provided by seven European institutions aimed to assess the prognostic value of VAV2 expression in ACC and to compare and integrate it with the Ki67 LI.

Materials and Methods

Immunostaining on formalin-fixed, paraffin-embedded ACC samples

We analyzed 171 adrenocortical tumor tissues from patients with ACC provided by seven ENS@T centers (three centers in Italy, n = 103 samples; two in The Netherlands, n = 42; one in France, n = 20; one in Germany, n = 6). A total of 145 samples were previously assembled in seven tissue microarrays (TMAs) with two or three cores per sample interspersed with normal human liver, kidney, and placenta tissues; 26 samples were available as full slides. Among the ACC samples, 157 were derived from primary tumors (male/female, 59:98; average age ± standard deviation [SD],

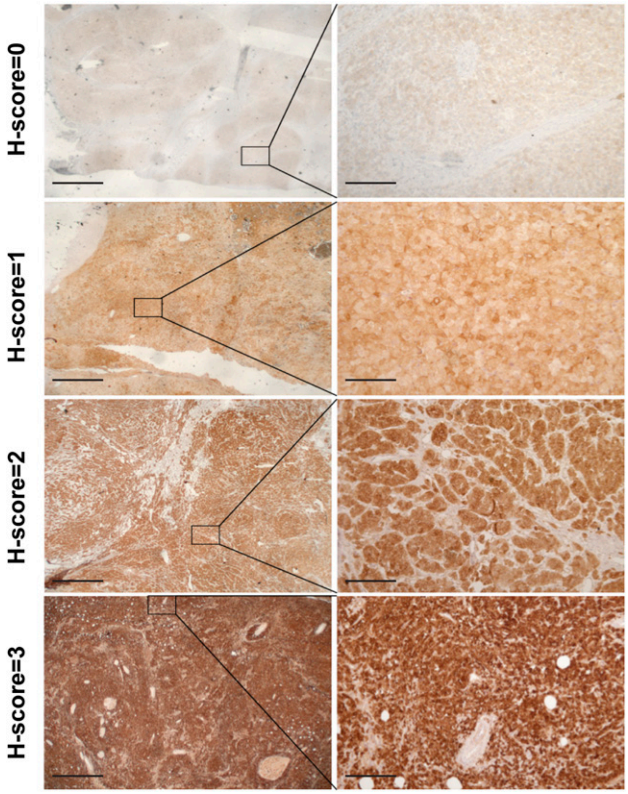

Figure 1. Examples of various intensities of VAV2 staining in ACC specimens. The H-score value is indicated for each image. Scale bar, 400 μm (images in left column); 50 μm (images in right column).

48.7 ± 15.2 years; average tumor size ± SD, 11.2 ± 5.4 cm; Supplemental Table 1)—six from local recurrences and eight from distant metastases (liver and lung). The diagnosis of ACC was made by established criteria based on clinical, biochemical, and morphological data (25). All clinical data were collected through the ENS@T database (registry.ensat.org). All patients gave informed consent and the study was approved by ethical committees from all participating institutions.

Immunohistochemical detection was performed on all samples using an indirect immunoperoxidase technique after high-temperature antigen retrieval in 0.01 M citrate buffer (pH 6.5) in a pressure cooker for 13 minutes. The primary antibody was a rabbit monoclonal antibody against the VAV2 protein (clone EP1067Y, catalog no. ab52640; Abcam) diluted 1:250 in 25% AB serum in phosphate-buffered saline and incubated for 1 hour at room temperature. Signal detection was performed with the ADVANCE HRP detection system (Dako) and 3,3′-diaminobenzidine chromogen according to the manufacturer's instructions. Nuclei were counterstained with Mayer hematoxylin for 3 minutes. As negative control, universal rabbit negative control (Dako) was used.

Immunostaining results were analyzed using a light microscope at high magnification. VAV2 staining intensity was evaluated independently by two investigators blinded to the clinical data (S.S. and I.S.). Cytoplasmic staining intensity was evaluated with a grading score of 0, 1, 2, or 3, corresponding to negative, weak, moderate, and strong intensity, respectively. The proportion of positive tumor cells was calculated for each specimen and set up to be scored 0, 0.1, 0.5, or 1, if 0%, 1% to 9%, 10% to 49%, or >50% of the tumor cells were positive for VAV2, respectively. A semiquantitative H-score was then calculated by multiplying the staining intensity grade by the proportion score (12, 24).

In all cases analyzed, the proportion of VAV2 positive cells was always >50%, so all intensity values were multiplied by a factor equal to 1 to yield the H-score. The cutoff point to separate samples in high or low VAV2 expression was between H-scores <2 and ≥2. Ki67 LI data assessed by the local pathologists in each expert center were retrieved from the ENS@T database. The Ki67 LI cutoff value used in this study to separate low LI and high LI groups was 20%.

Statistical analysis

Correlation analyses were performed using a χ^2 test for categorical variables. The interobserver agreement for the scoring system was evaluated using the Cohen κ coefficient and confirmed using Pearson correlation coefficient. The cutoff for strong agreement chosen for the κ coefficient was 0.81, and 0.75 was chosen for the Pearson coefficient (26). The comparison of clinical and histopathological characteristics was performed on GraphPad Prism 6.0 software using a nonparametric Mann-Whitney test (for two groups) and Kruskal-Wallis test with Dunn correction for multiple testing (for more than two groups), as appropriate. A P value <0.05 was considered to be

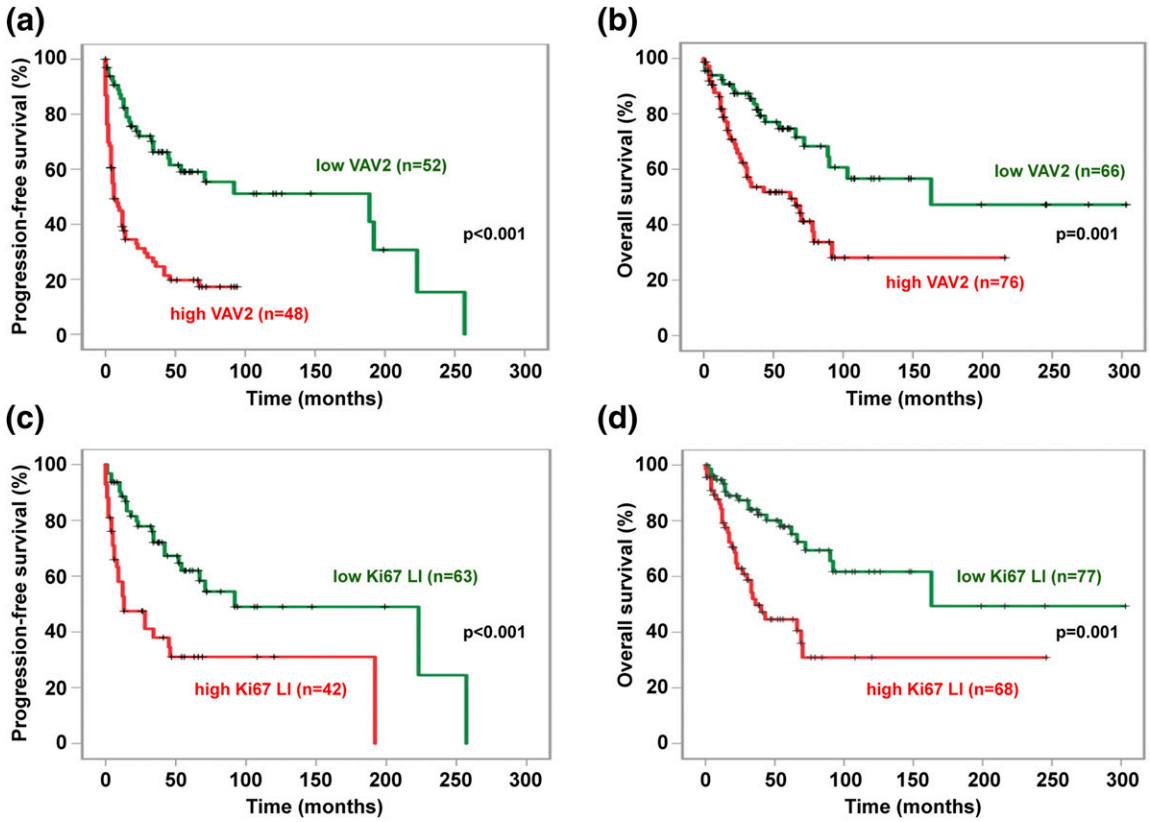

Figure 2. Correlation of VAV2 expression (H-score) and Ki67 LI with PFS and OS in our ACC series. (a) PFS (mean ± SD) in low VAV2 expression group (H-score, <2; green line), 127 ± 15.9 months; high VAV2 expression group (H-score, ≥2; red line), 25.7 ± 4.1 months (Kaplan-Meier $P <$ 0.001). (b) OS in low VAV2 expression group (H-score, <2; green line), 180 ± 22 months; high VAV2 expression group (H-score, ≥2; red line), 87.4 ± 13 months (Kaplan-Meier P = 0.001). (c) PFS in low Ki67 LI group (<20%; green line), 137 ± 17.9 months; high Ki67 LI group (≥20%; red line), 68.5 ± 14.3 months (Kaplan-Meier $P <$ 0.001). (d) OS in low Ki67 LI group (<20%; green line), 187.5 ± 22.9 months; high Ki67 LI group (≥20%; red line), 96.2 ± 17 months (Kaplan-Meier P = 0.001). The numbers of cases analyzed for each group are reported in parentheses.

statistically significant. Survival analysis for patients with ACC was calculated as described (24) using the Kaplan-Meier method, and differences between groups were assessed with log-rank and Cox proportional hazards statistics, using the SPSS software package (version 23.0.0 for Mac; IBM), after adjustment for sex, age, and tumor stage. Progression-free survival (PFS) was defined as time elapsed from primary resection of ACC to the first recurrence, locoregional or systemic. Overall survival (OS) was defined as time elapsed from primary resection of ACC to disease-related death or last follow-up visit. In the group of patients with R0 resection, OS data relative to VAV2 expression and Ki67 LI were available for 100 and 105 patients, respectively. Of those patients, 92 had both VAV2 and Ki67 LI OS data available. Viable cell data after *VAV2* knockdown were analyzed by one-way analysis of variance with Dunnett correction for multiple comparisons.

Results

VAV2 expression is a strong predictor of PFS and OS in patients with ACC

Examples of different VAV2 expression patterns in ACC are shown in Fig 1. An H-score was assigned to each sample, which took into consideration both staining intensity and the percentage of cells stained by the anti-VAV2 antibody. The interobserver agreement was very good, with the Cohen κ coefficient equal to 0.85 [95% confidence interval (CI), 0.72 to 0.89] and Pearson coefficient r equal to 0.90 (95% CI, 0.86 to 0.93; $P < 0.001$). In contrast to Ki67 staining, which is usually heterogeneous throughout a tumor, VAV2 expression was fairly equally distributed within a given tumor, with all samples presenting >50% stained cells. H-score heterogeneity among different TMA tissue cores belonging to the same tumors was limited, with a residual SD σ equal to 0.14 and an intraclass correlation coefficient α equal to 0.95 (95% CI, 0.92 to 0.97; Supplemental Fig. 1). The same homogenous distribution was also observed when whole tumor slides were analyzed (Fig 1).

VAV2 expression in the tumor was strongly correlated to both PFS [Fig 2(a)] and OS [Fig 2(b)], confirming the results of our previous study performed on

Table 1. Analysis of Parameters Correlated With PFS and OS in Univariate and Multivariate Analyses

	Univariate Analysis			Multivariate Analysis		
Survival	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
PFS						
Age (n = 113; n = 99)	1.00	0.98–1.02	0.77	0.99	0.98–1.01	0.91
Sex						
Female (n = 78; n = 69)						
Male (n = 35; n = 30)	1.19	0.69–2.04	0.52	1.17	0.64–2.13	0.59
Tumor stage						
I (n = 12; n = 8)						
II (n = 66; n = 61)	7.19	0.98–52.52	0.05	5.32	0.71–39.39	0.10
III (n = 23; n = 19)	6.29	0.81–48.65	0.07	5.03	0.64–39.65	0.12
IV (n = 11; n = 11)	27.51	3.40–222.11	0.002	14.65	1.74–122.96	0.01
VAV2 expression						
VAV2 low (H-score, 0–1; n = 52; n = 52)						
VAV2 high (H-score, 2–3; n = 48; n = 47)	2.80	1.57–4.98	<0.001	2.83	1.54–5.21	0.001
Ki67 LI						
Ki67 low (<20%; n = 63; n = 62)						
Ki67 high (≥20%; n = 42; n = 42)	2.77	1.58–4.86	<0.001	2.43	1.37–4.31	0.002
OS						
Age (n = 156; n = 74)	1.01	0.99–1.03	0.14	1.01	0.98–1.03	0.35
Sex						
Female (n = 98; n = 79)						
Male (n = 58; n = 45)	1.27	0.76–2.12	0.35	1.39	0.80–2.41	0.24
Tumor stage						
I (n = 12; n = 8)	3.71	0.50–27.60	0.2			
II (n = 72; n = 65)	3.71	0.50–27.60	0.2	3.77	0.49–28.84	0.20
III (n = 35; n = 29)	4.52	0.59–34.49	0.14	3.85	0.49–29.87	0.19
IV (n = 24; n = 22)	19.07	2.52–144.30	0.004	13.74	1.77–106	0.01
VAV2 expression						
VAV2 low (H-score, 0–1; n = 66; n = 60)						
VAV2 high (H-score, 2–3; n = 76; n = 64)	1.64	1.01–2.66	0.042	2.03	1.07–3.83	0.02
Ki67 LI						
Ki67 low (<20%; n = 77; n = 72)						
Ki67 high (≥20%; n = 68; n = 60)	2.94	1.67–5.19	<0.001	2.31	1.24–4.30	0.008

Numbers of cases taken into account for univariate and multivariate analysis, respectively, are indicated in parentheses for each variable.

Abbreviation: HR, hazard ratio.

an independent smaller cohort of patients with ACC (24). Patients with strong VAV2 expression had a 2.8-fold higher risk of experiencing a recurrence and 1.6-fold increased risk of dying.

No statistically significant difference existed for VAV2 expression in primary tumors and metastatic sites from the same patients (P = 0.67). The Ki67 LI was also a strong predictor of PFS [Fig 2(c)] and OS [Fig 2(d)], as reported in previous studies (14–18). Both VAV2 expression and Ki67 LI were strongly correlated with OS even in patients with R0 resection (Supplemental Fig. 2). VAV2 expression and Ki67 LI had a similar strong prognostic value for PFS and OS both in univariate and in multivariate analyses, taking into account patients' age, sex, and tumor stage (Table 1).

Combined assessment of VAV2 expression and Ki67 LI improves prognostic power

In general, a significant correlation existed between Ki67 LI and VAV2 expression in our ACC cohort (Supplemental Fig. 3). A strong correlation also existed when Ki67 LI and VAV2 expression were considered as categorical (low *vs* high) variables (χ^2 = 6.18; P = 0.01). However, in several cases, these two parameters were

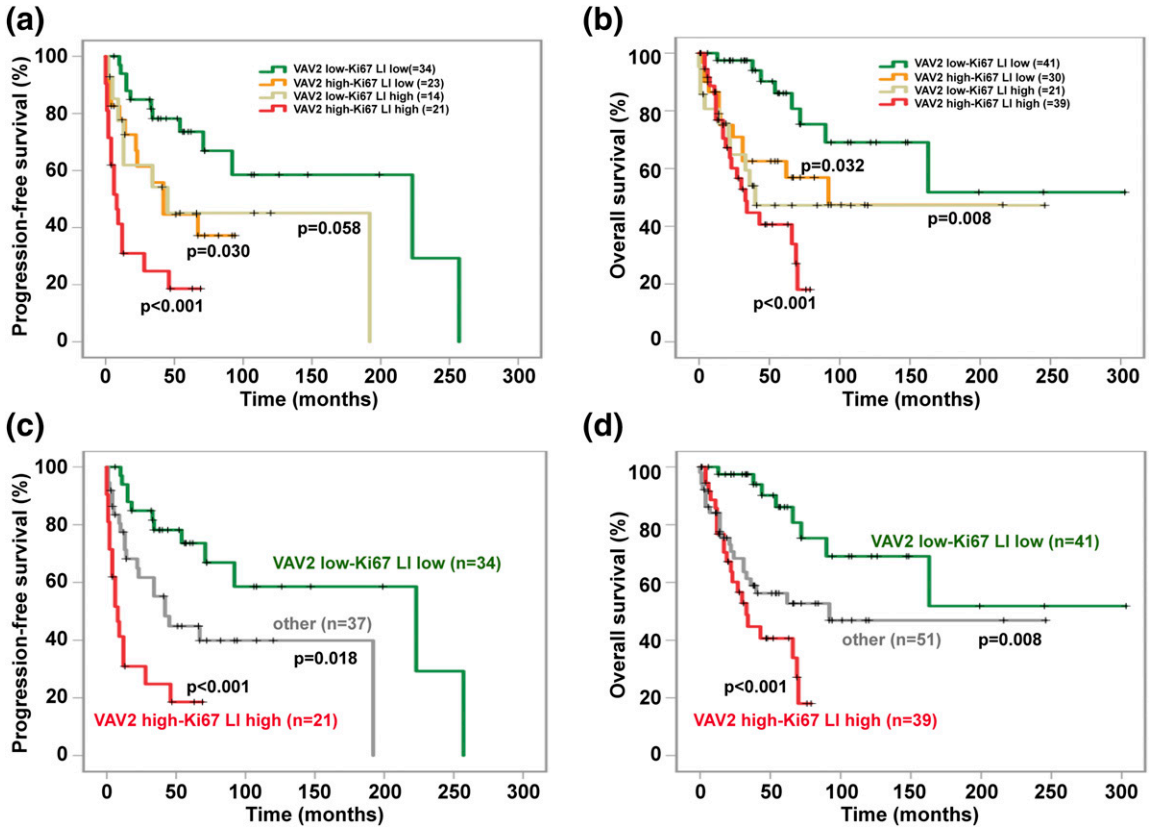

Figure 3. Correlation of combined VAV2 expression (H-score) and Ki67 LI with PFS and OS in our ACC series. (a) PFS (mean ± SD) in low VAV2 expression (H-score, <2) and low Ki67 LI group (<20%; green line), 159.7 ± 23.2 months; high VAV2 expression (H-score, ≥2)-low Ki67 LI group (<20%; yellow line), 50.7 ± 8.4 months; low VAV2 expression (H-score, <2)-high Ki67 LI group (≥20%; pale green line), 96.6 ± 26.3 months; high VAV2 expression (H-score, ≥2)-high Ki67 LI group (≥ 20%; red line), 20.8±5.8 months. Compared with the low VAV2-low Ki67 LI group: the high VAV2-low Ki67 LI hazard ratio (HR) was 2.55 (95% CI, 1.09–5.97; P = 0.030); low VAV2-high Ki67 LI HR was 2.46 (95% CI, 0.97–6.23; P = 0.058); high VAV2-high Ki67 LI HR was 6.75 (95% CI, 2.97–15.31; $P < 0.001$) by the Kaplan-Meier method. (b) OS (mean ± SD) in low VAV2 expression (H-score, <2)-low Ki67 LI group (<20%; green line), 203.7 ± 29.6 months; high VAV2 expression (H-score, ≥2)-low Ki67 LI group (<20%; yellow line), 120.4 ± 20.5 months; low VAV2 expression (H-score, <2)-high Ki67 LI group (≥20%; pale green line), 126 ± 26.7 months; high VAV2 expression (H-score, ≥2)-high Ki67 LI group (≥20%; red line), 41.6 ± 5.1 months. Compared with the low VAV2-low Ki67 LI group: high VAV2-low Ki67 LI HR was 2.66 (95% CI, 1.08–6.52; P = 0.032); low VAV2-high Ki67 LI HR was 3.51 (95% CI, 1.38–8.91; P = 0.008); high VAV2-high Ki67 LI HR was 5.38 (95% CI, 2.33–12.40; $P < 0.001$) by the Kaplan-Meier method. (c) PFS (mean ± SD) in low VAV2 expression (H-score, <2)-low Ki67 LI group (<20%; green line), 159.7 ± 23.2 months; high VAV2 expression (H-score, ≥2)-high Ki67 LI group (≥20%; red line), 20.8 ± 5.8 months; all other patients with dissociated VAV2 expression-Ki67 LI group (gray line), 90.3 ± 15.7 months. Compared with the low VAV2-low Ki67 LI group: other HR was 2.51 (95% CI, 1.17–5.39; P = 0.018); high VAV2-high Ki67 LI group HR was 6.75 (95% CI, 2.97–15.31; $P < 0.001$) by the Kaplan-Meier method. (d) OS (mean ± SD) in low VAV2 expression (H-score, <2)-low Ki67 LI group (<20%; green line), 203.7 ± 29.6 months; high VAV2 expression (H-score, ≥2)-high Ki67 LI group (≥20%; red line), 41.6 ± 5.1 months; all other patients with dissociated VAV2 expression-Ki67 LI group (gray line), 130.3 ± 18.1 months. Compared with the low VAV2-low Ki67 LI group: other HR was 2.99 (95% CI, 1.32–6.73; P = 0.008); high VAV2-high Ki67 LI group HR was 5.38 (95% CI, 2.33–12.40; $P < 0.001$) by the Kaplan-Meier method. The numbers of cases analyzed for each group are reported in parentheses.

dissociated, with one value being elevated and the other low in the same tumor. Remarkably, in those patients, PFS and OS were intermediate between the high-risk (high VAV2 expression and high Ki67 LI) and the low-risk groups [low VAV2 expression and low Ki67 LI; Fig 3(a) and 3(b)].

Merging the groups with high VAV2/low Ki67 LI and low VAV2/high Ki67 LI and comparing them with the high VAV2/high Ki67 LI and low VAV2/low Ki67 LI groups identified three classes of patients with very different PFS (mean ± SD: 159.7 ± 23.2, 90.3 ± 15.7, and 20.8 ± 5.8 months, respectively) and OS [mean ± SD: 203.7 ± 29.6, 130.3 ± 29.6, and 41.6 ± 5.1 months, respectively; Fig 3(c) and 3(d)]. This type of stratification maintained a strong prognostic value even in R0 patients (Supplemental Fig. 4). Remarkably, when considering the high-risk group apart from all other patients with ACC, a very strong correlation existed with OS in the whole cohort [Fig 4(a)] and with both PFS and OS in R0 patients [Fig 4(b) and 4(c)]. Furthermore, isolated high VAV2 expression or high Ki67 LI showed a prediction value for worse PFS and OS that was slightly lower compared with the combination of both high VAV2 expression and high Ki67 LI [PFS: 22 months, hazard ratio (HR), 0.67 for VAV2, and 28 months, HR, 0.66 for Ki67 LI *vs* 9 months for the combination; OS: 66 months, HR, 0.73 for VAV2, and 40 months, HR, 0.82 for Ki67 LI *vs* 33 months for the combination].

Discussion

The prognosis of patients with ACC is variable and poorly predictable. A recent, large multicenter ENS@T study has shown that the KI67 LI is the most powerful parameter predicting disease recurrence and survival in patients with ACC after complete tumor resection (18). The Ki67 LI has been integrated with the combined evaluation of morphological parameters (*i.e.*, number of mitoses/presence of necrosis) in the newly introduced Helsinki score, which reportedly more accurately predicts recurrence in ACC (8, 27). However, even if Ki67 LI assessment is routinely performed in diagnostic

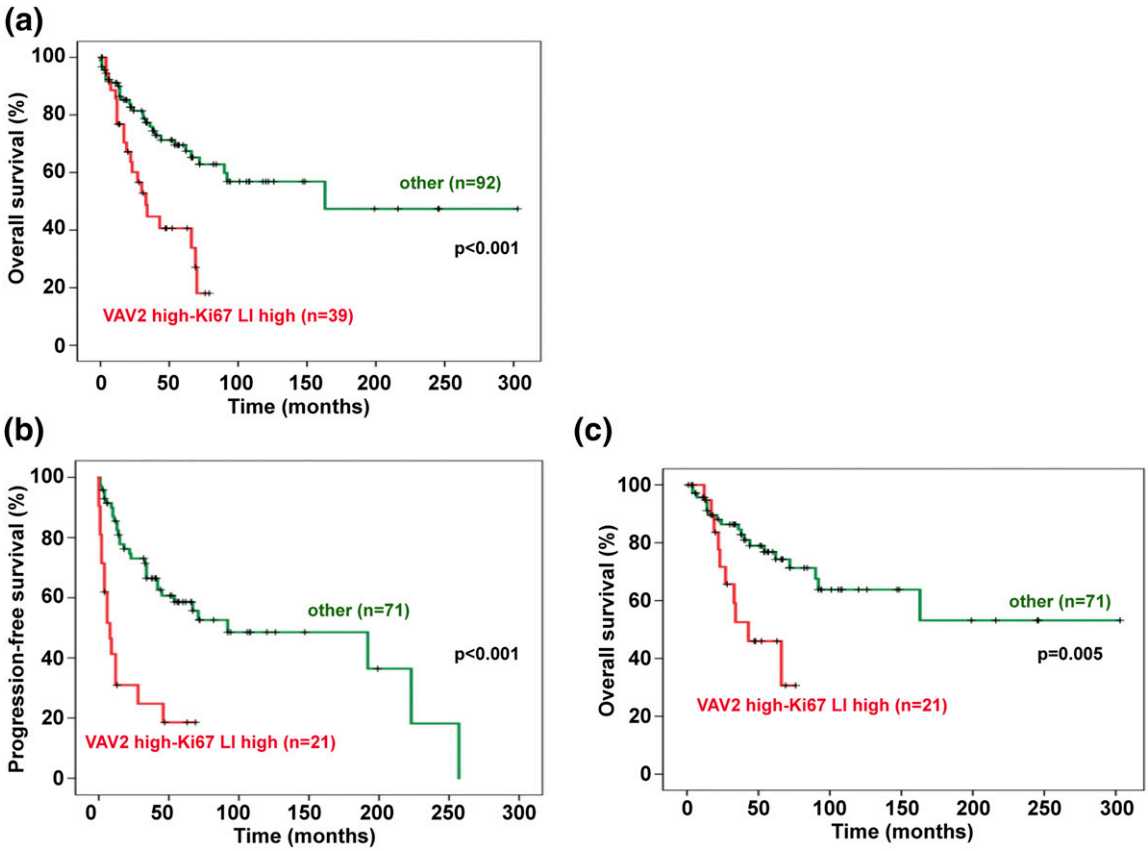

Figure 4. Prognosis of the high-risk group (high VAV2 expression-high Ki67 LI) *vs* other patients with ACC. (a) OS (mean ± SD) in the whole cohort of patients with ACC for the high VAV2 expression (H-score, ≥2)-high Ki67 LI group (≥20%; red line) was 41.5±5 months; for all other patients (green line), OS was 175.5 ± 19.8 months (Kaplan-Meier $P < 0.001$). (b) PFS (mean ± SD) in R0 patients for the high VAV2 expression (H-score, ≥2)-high Ki67 LI group (≥20%; red line), 20.8 ± 5.8 months; for all other patients (green line), PFS was 127.3 ± 15.7 months (Kaplan-Meier $P < 0.001$). (c) OS (mean ± SD) in R0 patients for the high VAV2 expression (H-score, ≥2)-high Ki67 LI group (≥20%; red line) was 47.5±6 months; OS all other patients (green line) was 194.8 ± 21.7 months (Kaplan-Meier $P = 0.005$). The number of cases analyzed for each group are reported in parentheses.

pathology laboratories for many neoplastic disorders, its standardization and reproducibility have been questioned for many tumor types, including ACC (19). It is important, therefore, to identify other molecular markers that can complement the Ki67 LI to obtain a more accurate stratification of the risk of recurrence in patients with ACC. In this perspective, molecular prognostic indicators derived from genomic studies are very promising (3, 28, 29), but for routine implementation, they suffer from the important drawback that, at least at the present state of technology, frozen tumor material is required. On the other hand, prognostic value of circulating markers of malignancy awaits validation in large cohorts of patients with ACC (30–33).

We have recently shown that VAV2 overexpression is an essential driver of cell invasion in conditions of increased SF-1 dosage through its guanine nucleotide exchange factor activity for the small GTPases Rac1 and Cdc42 (24). Those data directly link VAV2 with the potential mechanism of malignancy consisting of increased cellular invasiveness. In the current study, we extended the previous study to a large European cohort of patients with ACC and show that the tumor VAV2 H-score is significantly correlated to PFS and OS. The combined assessment of VAV2 expression and Ki67 LI improves patient risk stratification, with cases presenting high Ki67 LI but low VAV2 expression having significantly longer PFS and OS compared with patients with concordant high-risk parameters. In our study, VAV2 H-score assessment, which was mainly performed on TMA tissue cores, was associated with an excellent intratumoral reproducibility and is, in principle, less prone to intra- and interobserver variability, although further work is needed to specifically address this question on an even larger number of cases. These results show that immunohistochemical assessment of VAV2 expression may usefully complement the measurement of the Ki67 LI for prognostic stratification of patients with ACC.

Acknowledgments

Address all correspondence and requests for reprints to: Enzo Lalli, MD, Institut de Pharmacologie Moléculaire et Cellulaire CNRS UMR7275, 660 route des Lucioles - Sophia Antipolis, 06560 Valbonne, France. E-mail: ninino@ipmc.cnrs.fr or Silviu Sbiera, PhD, Medizinische Klinik und Poliklinik I - University of Würzburg Endokrinologie Forschung ZIM, A4.-3.949 Oberdürrbacherstrasse 6 97080 Würzburg, Germany. E-mail: Sbiera_S@ukw.de.

This work was supported by the Else Kröner-Fresenius-Stiftung Grant 2016_A96 and by a fellowship from the "Novartis-Stiftung für theraputische Forschung" to S.S.; Italian Association for Cancer Research Grants IG/14820/2013 (to M.P.), 14411 (to M.T.), and IG/17691/2015 (to M.L.); ERA-NET "E-Rare" Grant 01GM1407B (to M.F.); French National Research Agency (ANR) through the LOCALDO (Grant ANR-15-CE14-0017-01) and "Investments for the Future" Labex SIGNALIFE (Grant ANR-11-LABX-0028-01) (to E.L.). C.R. was a recipient of Ville de Nice and Fondation de France (Grant 00057927) postdoctoral fellowships.

Disclosure Summary: The authors have nothing to disclose.

References

1. Else T, Kim AC, Sabolch A, Raymond VM, Kandathil A, Caoili EM, Jolly S, Miller BS, Giordano TJ, Hammer GD. Adrenocortical carcinoma. *Endocr Rev*. 2014;**35**(2):282–326.
2. Ronchi CL, Kroiss M, Sbiera S, Deutschbein T, Fassnacht M. EJE prize 2014: current and evolving treatment options in adrenocortical carcinoma: where do we stand and where do we want to go? *Eur J Endocrinol*. 2014;**171**(1):R1–R11.
3. de Reyniès A, Assié G, Rickman DS, Tissier F, Groussin L, René-Corail F, Dousset B, Bertagna X, Clauser E, Bertherat J. Gene expression profiling reveals a new classification of adrenocortical tumors and identifies molecular predictors of malignancy and survival. *J Clin Oncol*. 2009;**27**(7):1108–1115.
4. Giordano TJ, Kuick R, Else T, Gauger PG, Vinco M, Bauersfeld J, Sanders D, Thomas DG, Doherty G, Hammer G. Molecular classification and prognostication of adrenocortical tumors by transcriptome profiling. *Clin Cancer Res*. 2009;**15**(2):668–676.
5. Assié G, Letouzé E, Fassnacht M, Jouinot A, Luscap W, Barreau O, Omeiri H, Rodriguez S, Perlemoine K, René-Corail F, Elarouci N, Sbiera S, Kroiss M, Allolio B, Waldmann J, Quinkler M, Mannelli M, Mantero F, Papathomas T, De Krijger R, Tabarin A, Kerlan V, Baudin E, Tissier F, Dousset B, Groussin L, Amar L, Clauser E, Bertagna X, Ragazzon B, Beuschlein F, Libé R, de Reyniès A, Bertherat J. Integrated genomic characterization of adrenocortical carcinoma. *Nat Genet*. 2014;**46**(6):607–612.
6. Zheng S, Cherniack AD, Dewal N, Moffitt RA, Danilova L, Murray BA, Lerario AM, Else T, M TA, Ciriello G, Kim S, Assie G, Morozova O, Akbani R, Shih J, Hoadley KA, Choueiri TK, Waldmann J, Mete O, Robertson AG, Wu HT, Raphael BJ, Shao L, Meyerson M, Demeure MJ, Beuschlein F, Gill AJ, Sidhu SB, Almeida MQ, Fragoso MC, Cope LM, Kebebew E, Habra MA, Whitsett TG, Bussey KJ, Rainey WE, Asa SL, Bertherat J, Fassnacht M, Wheeler DA, Hammer GD, Giordano TJ, Verhaak RG; Cancer Genome Atlas Research Network. Comprehensive pan-genomic characterization of adrenocortical carcinoma [published correction appears in Cancer Cell. 2016;30(2):363. *Cancer Cell*. 2016;**29**(5):723–736.
7. Papotti M, Libè R, Duregon E, Volante M, Bertherat J, Tissier F. The Weiss score and beyond–histopathology for adrenocortical carcinoma. *Horm Cancer*. 2011;**2**(6):333–340.
8. Pennanen M, Heiskanen I, Sane T, Remes S, Mustonen H, Haglund C, Arola J. Helsinki score-a novel model for prediction of metastases in adrenocortical carcinomas. *Hum Pathol*. 2015;**46**(3):404–410.
9. Volante M, Sperone P, Bollito E, Frangipane E, Rosas R, Daffara F, Terzolo M, Berruti A, Papotti M. Matrix metalloproteinase type 2 expression in malignant adrenocortical tumors: diagnostic and prognostic significance in a series of 50 adrenocortical carcinomas. *Mod Pathol*. 2006;**19**(12):1563–1569.
10. Ronchi CL, Sbiera S, Kraus L, Wortmann S, Johanssen S, Adam P, Willenberg HS, Hahner S, Allolio B, Fassnacht M. Expression of excision repair cross complementing group 1 and prognosis in adrenocortical carcinoma patients treated with platinum-based chemotherapy. *Endocr Relat Cancer*. 2009;**16**(3):907–918.
11. Fenske W, Völker HU, Adam P, Hahner S, Johanssen S, Wortmann S, Schmidt M, Morcos M, Müller-Hermelink HK, Allolio B, Fassnacht M. Glucose transporter GLUT1 expression is an stage-independent

predictor of clinical outcome in adrenocortical carcinoma. *Endocr Relat Cancer*. 2009;**16**(3):919–928.
12. Sbiera S, Schmull S, Assie G, Voelker HU, Kraus L, Beyer M, Ragazzon B, Beuschlein F, Willenberg HS, Hahner S, Saeger W, Bertherat J, Allolio B, Fassnacht M. High diagnostic and prognostic value of steroidogenic factor-1 expression in adrenal tumors. *J Clin Endocrinol Metab*. 2010;**95**(10):E161–E171.
13. Volante M, Terzolo M, Fassnacht M, Rapa I, Germano A, Sbiera S, Daffara F, Sperone P, Scagliotti G, Allolio B, Papotti M, Berruti A. Ribonucleotide reductase large subunit (RRM1) gene expression may predict efficacy of adjuvant mitotane in adrenocortical cancer. *Clin Cancer Res*. 2012;**18**(12):3452–3461.
14. McNicol AM, Struthers AL, Nolan CE, Hermans J, Haak HR. Proliferation in adrenocortical tumors: correlation with clinical outcome and p53 status. *Endocr Pathol*. 1997;**8**(1):29–36.
15. Terzolo M, Boccuzzi A, Bovio S, Cappia S, De Giuli P, Alì A, Paccotti P, Porpiglia F, Fontana D, Angeli A. Immunohistochemical assessment of Ki-67 in the differential diagnosis of adrenocortical tumors. *Urology*. 2001;**57**(1):176–182.
16. Morimoto R, Satoh F, Murakami O, Suzuki T, Abe T, Tanemoto M, Abe M, Uruno A, Ishidoya S, Arai Y, Takahashi K, Sasano H, Ito S. Immunohistochemistry of a proliferation marker Ki67/MIB1 in adrenocortical carcinomas: Ki67/MIB1 labeling index is a predictor for recurrence of adrenocortical carcinomas. *Endocr J*. 2008; **55**(1):49–55.
17. Duregon E, Molinaro L, Volante M, Ventura L, Righi L, Bolla S, Terzolo M, Sapino A, Papotti MG. Comparative diagnostic and prognostic performances of the hematoxylin-eosin and phosphohistone H3 mitotic count and Ki-67 index in adrenocortical carcinoma. *Mod Pathol*. 2014;**27**(9):1246–1254.
18. Beuschlein F, Weigel J, Saeger W, Kroiss M, Wild V, Daffara F, Libé R, Ardito A, Al Ghuzlan A, Quinkler M, Oßwald A, Ronchi CL, de Krijger R, Feelders RA, Waldmann J, Willenberg HS, Deutschbein T, Stell A, Reincke M, Papotti M, Baudin E, Tissier F, Haak HR, Loli P, Terzolo M, Allolio B, Müller HH, Fassnacht M. Major prognostic role of Ki67 in localized adrenocortical carcinoma after complete resection. *J Clin Endocrinol Metab*. 2015;**100**(3): 841–849.
19. Papathomas TG, Pucci E, Giordano TJ, Lu H, Duregon E, Volante M, Papotti M, Lloyd RV, Tischler AS, van Nederveen FH, Nose V, Erickson L, Mete O, Asa SL, Turchini J, Gill AJ, Matias-Guiu X, Skordilis K, Stephenson TJ, Tissier F, Feelders RA, Smid M, Nigg A, Korpershoek E, van der Spek PJ, Dinjens WN, Stubbs AP, de Krijger RR. An international Ki67 reproducibility study in adrenal cortical carcinoma. *Am J Surg Pathol*. 2016;**40**(4):569–576.
20. Lalli E. Adrenocortical development and cancer: focus on SF-1. *J Mol Endocrinol*. 2010;**44**(6):301–307.
21. Doghman M, Karpova T, Rodrigues GA, Arhatte M, De Moura J, Cavalli LR, Virolle V, Barbry P, Zambetti GP, Figueiredo BC, Heckert LL, Lalli E. Increased steroidogenic factor-1 dosage triggers adrenocortical cell proliferation and cancer. *Mol Endocrinol*. 2007;**21**(12):2968–2987.
22. Doghman M, Figueiredo BC, Volante M, Papotti M, Lalli E. Integrative analysis of SF-1 transcription factor dosage impact on genome-wide binding and gene expression regulation. *Nucleic Acids Res*. 2013;**41**(19):8896–8907.
23. Bustelo XR. Vav family exchange factors: an integrated regulatory and functional view. *Small GTPases*. 2014;**5**(2):9.
24. Ruggiero C, Doghman-Bouguerra M, Sbiera S, Sbiera I, Parsons M, Ragazzon B, Morin A, Robidel R, Favier J, Bertherat J, Fassnacht M, Lalli E. Dosage-dependent regulation of VAV2 expression by steroidogenic factor-1 drives adrenocortical carcinoma cell invasion. *Sci Signal*. 2017;**10**(469).
25. Fassnacht M, Kroiss M, Allolio B. Update in adrenocortical carcinoma. *J Clin Endocrinol Metab*. 2013;**98**(12):4551–4564.
26. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics*. 1977;**33**(1):159–174.
27. Duregon E, Cappellesso R, Maffeis V, Zaggia B, Ventura L, Berruti A, Terzolo M, Fassina A, Volante M, Papotti M. Validation of the prognostic role of the "Helsinki Score" in 225 cases of adrenocortical carcinoma. *Hum Pathol*. 2017;**62**:1–7.
28. Fragoso MC, Almeida MQ, Mazzuco TL, Mariani BM, Brito LP, Gonçalves TC, Alencar GA, Lima LdeO, Faria AM, Bourdeau I, Lucon AM, Freire DS, Latronico AC, Mendonca BB, Lacroix A, Lerario AM. Combined expression of BUB1B, DLGAP5, and PINK1 as predictors of poor outcome in adrenocortical tumors: validation in a Brazilian cohort of adult and pediatric patients. *Eur J Endocrinol*. 2012;**166**(1):61–67.
29. Jouinot A, Assie G, Libe R, Fassnacht M, Papathomas T, Barreau O, de la Villeon B, Faillot S, Hamzaoui N, Neou M, Perlemoine K, Rene-Corail F, Rodriguez S, Sibony M, Tissier F, Dousset B, Sbiera S, Ronchi C, Kroiss M, Korpershoek E, de Krijger R, Waldmann J, K D, Bartsch, Quinkler M, Haissaguerre M, Tabarin A, Chabre O, Sturm N, Luconi M, Mantero F, Mannelli M, Cohen R, Kerlan V, Touraine P, Barrande G, Groussin L, Bertagna X, Baudin E, Amar L, Beuschlein F, Clauser E, Coste J, Bertherat J. DNA methylation is an independent prognostic marker of survival in adrenocortical cancer. *J Clin Endocrinol Metab*. 2017;**102**(3):923–932.
30. Chabre O, Libé R, Assie G, Barreau O, Bertherat J, Bertagna X, Feige JJ, Cherradi N. Serum miR-483-5p and miR-195 are predictive of recurrence risk in adrenocortical cancer patients. *Endocr Relat Cancer*. 2013;**20**(4):579–594.
31. Patel D, Boufraqech M, Jain M, Zhang L, He M, Gesuwan K, Gulati N, Nilubol N, Fojo T, Kebebew E. MiR-34a and miR-483-5p are candidate serum biomarkers for adrenocortical tumors. *Surgery*. 2013;**154**(6):1224–1228, discussion 1229.
32. Szabó DR, Luconi M, Szabó PM, Tóth M, Szücs N, Horányi J, Nagy Z, Mannelli M, Patócs A, Rácz K, Igaz P. Analysis of circulating microRNAs in adrenocortical tumors. *Lab Invest*. 2014; **94**(3):331–339.
33. Liu-Chittenden Y, Patel D, Gaskins K, Giordano TJ, Assie G, Bertherat J, Kebebew E. Serum RARRES2 Is a prognostic marker in patients with adrenocortical carcinoma. *J Clin Endocrinol Metab*. 2016;**101**(9):3345–3352.