CLINICAL STUDY

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

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Abstract

Background: A recent microarray study identified a set of genes whose combined expression patterns were predictive of poor outcome in a cohort of adult adrenocortical tumors (ACTs). The difference between the expression values measured by qRT-PCR of *DLGAP5* and *PINK1* genes was the best molecular predictor of recurrence and malignancy. Among the adrenocortical carcinomas, the combined expression of *BUB1B* and *PINK1* genes was the most reliable predictor of overall survival. The prognostic and molecular heterogeneity of ACTs raises the need to study the applicability of these molecular markers in other cohorts.

Objective: To validate the combined expression of *BUB1B*, *DLGAP5*, and *PINK1* as outcome predictor in ACTs from a Brazilian cohort of adult and pediatric patients.

Patients and methods: *BUB1B*, *DLGAP5*, and *PINK1* expression was assessed by quantitative PCR in 53 ACTs from 52 patients – 24 pediatric and 28 adults (one pediatric patient presented a bilateral asynchronous ACT).

Results: *DLGAP5–PINK1* and *BUB1B–PINK1* were strong predictors of disease-free survival and overall survival, respectively, among adult patients with ACT. In the pediatric cohort, these molecular predictors were only marginally associated with disease-free survival but not with overall survival.

Conclusion: This study confirms the prognostic value of the combined expression of *BUB1B*, *DLGAP5*, and *PINK1* genes in a Brazilian group of adult ACTs. Among pediatric ACTs, other molecular predictors of outcome are required.

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Introduction

Adrenocortical tumors (ACTs) are common neoplasms with a prevalence of at least 3% in a population over the age of 50 years (1). Adrenocortical carcinoma (ACC) is a rare and aggressive malignancy, with a 5-year survival lower than 35% in most populations (2). In adults, the differential diagnosis between an adrenocortical adenoma (ACA) and a localized ACC rely on the presence of at least three of nine histological Weiss criteria (3). However, in some cases, the diagnosis of malignancy is not straightforward. ACCs in children and adolescents constitute only 0.2% of all pediatric malignancies (4, 5). A remarkably high annual incidence of ACTs has been reported in children younger than 15 years from

Southern Brazil, where a high prevalence of a germline mutation of the *TP53* tumor suppressor (p.R337H) was identified (6, 7). In contrast to adults, pediatric ACTs with apparently poor prognosis based on the histopathological features often have a better clinical outcome (8).

ACTs are heterogeneous neoplasms with incompletely understood pathogenesis. Different transcriptome analyses confirmed *IGF2* overexpression in the majority of adult ACCs (9, 10). In contrast, a single microarray analysis of pediatric ACTs did not discriminate ACAs and ACCs using unsupervised clustering (11). *IGF2* and *FGFR4* were similarly expressed in both benign and malignant pediatric ACTs (11, 12). Interestingly, *IGF1R* gene, whose product mediates the mitogenic effects

of IGF2, was overexpressed in metastatic pediatric ACCs (12, 13).

Recently, de Reyniès *et al.* (14) identified genes in a microarray study that robustly discriminated adult ACAs and ACCs and identified two clusters of ACCs with distinct outcomes. They identified and validated that the combined expression levels of three genes were strong predictors of overall survival and disease-free survival. Accordingly, the difference between the expression values of discs large associated protein 7 (*DLGAP5*) and PTEN-induced putative kinase 1 (*PINK1*; ΔC_T *DLGAP5*– ΔC_T *PINK1*) was a strong predictor of recurrence and malignancy and the difference between the expression values of budding uninhibited by benzimidazoles 1 homolog beta (*BUB1B*) and *PINK1* (ΔC_T *BUB1B*– ΔC_T *PINK1*) was a reliable predictor of overall survival (14). These new molecular markers identified in a European population of patients may provide important predictors for the diagnosis and the management of adult ACTs. However, it needs to be validated in other groups of adult patients and studied in pediatric ACTs, whose molecular pathogenesis is clearly different from adult tumors (5).

In the current study, we investigated the prognostic value of the combined expression of *DLGAP5*, *BUB1B*, and *PINK1* genes in an adult cohort of Brazilian patients with ACTs. In addition, we examined for the first time whether the same molecular markers can predict outcome in pediatric ACTs.

Materials and methods

The study was approved by the Ethics Committee of the Hospital das Clínicas, Sao Paulo, Brazil, and informed written consent was obtained from all patients and/or parents. Clinical and pathological data were retrospectively recorded. The study cohort consisted of 52 patients (24 pediatric and 28 adults) evaluated in our Institution from 1990 to 2010 (see Tables 1 and 2). The mean follow-up period in pediatric and adult groups was 80.4 and 65.8 months respectively. Endocrine work-up was performed in all patients.

In the pediatric group, 20 out of 24 cases were classified as ACC according to Weiss criteria (Weiss score ≥ 3). However, only eight patients presented an advanced tumor stage (MacFarlane III or IV) at diagnosis and/or poor clinical outcome. Among adult ACTs, 14 cases were classified as ACAs (Weiss score < 3) and 14 as ACCs (Weiss score ≥ 3). Metastases were diagnosed by systematic imaging investigations mainly abdominal and chest computed tomography (CT) scans; bone scintigraphy in appropriated cases, magnetic resonance imaging, and more recently in selected cases with positron emission CT scan. All patients were submitted to unilateral adrenalectomy except one pediatric patient who presented a bilateral asynchronous ACT (15). Histopathological diagnosis, tumor weight, size, and Weiss scores were determined for each tumor specimen. Death related to ACC progression occurred in five pediatric and nine adult patients, within

Table 1 Clinical and molecular data of pediatric adrenocortical tumor patients.

Patient	Sex	Age (years)	Size (cm)	Stage ^a	Weiss	<i>BUB1B</i> – <i>PINK1</i>	<i>DLGAP5</i> – <i>PINK1</i>	Recurrence/metastasis	DFS (months)	Outcome	Follow-up (months)
1	F	0.90	6.00	II	3	8.2390	7.9830	No	99.00	Alive	99.00
2	F	0.98	8.50	III	5	–1.6067	0.2600	On follow-up	6.00	Alive	65.57
3	F	1.25	3.70	I	5	3.9733	4.3300	No	24.80	Alive	24.80
4	F	1.58	4.00	I	3	3.5567	6.6150	No	26.15	Alive	26.15
6	M	1.72	4.00	I	4	–1.8467	–0.3433	No	164.10	Alive	164.10
7	M	2.00	6.50	II	4	0.0740	–2.7520	No	174.00	Alive	174.00
9	F	2.00	5.00	I	4	–0.3690	0.7790	No	84.00	Alive	84.00
10	F	2.08	4.00	I	4	–1.2450	–1.3540	No	79.00	Alive	60.00
11	F	2.10	3.00	I	1	–1.5480	0.3840	No	148.00	Alive	148.00
13	F	2.42	3.80	I	5	–1.3717	1.0333	No	74.60	Alive	74.60
14	F	2.44	2.50	I	N/A	5.4767	6.7067	No	72.40	Alive	72.40
15	F	2.52	5.20	II	7	–1.9200	1.1100	No	100.77	Alive	100.77
16	M	2.59	2.00	I	5	4.3000	5.6300	No	155.83	Alive	155.83
17	F	2.87	3.50	I	2	0.9760	0.4740	No	167.17	Alive	167.17
19	M	3.20	8.00	III	7	1.1750	–1.3310	On follow-up	3.00	CRD	12.00
20	M	4.88	8.50	III	6	–1.0883	1.6583	On follow-up	15.00	Alive	127.97
21	F	6.07	7.00	II	7	–2.2100	0.6633	On follow-up	10.00	CRD	15.80
22	F	9.32	6.00	IV	7	–1.5883	0.1117	At diagnosis	–	CRD	15.33
23	M	9.41	4.50	I	2	6.7530	6.7770	No	96.00	Alive	96.00
25	F	15.17	20.00	IV	7	–1.1400	0.9333	At diagnosis	–	CRD	21.50
26	F	16.70	4.00	I	3	6.1733	8.0100	No	25.20	Alive	25.20
27	F	17.20	15.00	II	4	5.0800	5.7830	No	115.00	Alive	115.00
28	M	17.32	9.00	III	5	3.4550	11.2000	No	60.00	Alive	60.00
29	F	17.47	10.00	IV	8	–0.9110	–2.7570	At diagnosis	–	CRD	6.93

DFS, disease-free survival; CRD, cancer-related death.

^aAccording to MacFarlane system.

Table 2 Clinical and molecular data of adult adrenocortical tumor patients.

Patient	Sex	Age (years)	Size (cm)	Stage ^a	Weiss	<i>BUB1B</i> – <i>PINK1</i>	<i>DLGAP5</i> – <i>PINK1</i>	Recurrence/metastasis	DFS (months)	Out-come	Follow-up (months)
30	F	19.00	13.00	III	8	–2.4760	–3.2280	On follow-up	8.00	CRD	10.00
31	F	22.00	13.00	IV	7	–0.5133	1.3000	At diagnosis	–	CRD	12.00
32	F	22.44	14.50	II	8	–1.6100	–0.5800	On follow-up	1.00	CRD	15.00
33	F	22.81	6.00	III	5	1.9060	–0.7210	On follow-up	4.00	Alive	14.77
34	F	22.82	14.00	IV	6	1.3833	3.2200	At diagnosis	–	CRD	15.20
35	F	23.54	4.00	I	2	4.2733	7.4500	No	28.40	Alive	28.40
36	F	24.00	3.00	I	2	6.0620	4.9720	No	159.00	Alive	159.00
37	F	26.00	5.50	II	1	6.2310	6.2500	No	64.00	Alive	64.00
38	F	26.88	2.50	I	2	7.5200	9.3233	No	90.97	Alive	90.97
41	F	28.88	12.00	IV	4	–7.3617	–2.5000	At diagnosis	–	CRD	28.30
42	F	29.00	5.00	I	0	7.6050	7.1930	No	160.00	Alive	160.00
43	M	29.61	19.00	IV	8	–4.1533	–1.1867	At diagnosis	–	CRD	8.00
44	M	30.00	9.60	III	4	0.2250	–3.0130	On follow-up	4.00	CRD	12.00
45	F	30.48	12.00	III	6	–0.2467	1.7267	On follow-up	4.00	CRD	12.00
46	F	35.00	2.00	I	3	5.3330	6.6650	No	102.00	Alive	102.00
47	F	35.56	3.00	I	0	7.5833	9.3633	No	54.12	Alive	54.17
48	F	35.73	6.00	II	1	1.7767	4.0000	No	121.43	Alive	121.43
50	F	37.60	2.50	I	2	7.3200	9.7267	No	202.83	Alive	202.83
51	F	39.00	2.50	I	0	5.7280	6.0770	No	93.00	Alive	93.00
52	F	41.00	2.80	I	1	5.1300	4.6500	No	143.00	Alive	143.00
53	F	41.36	1.80	I	0	6.4667	8.2000	No	43.17	Alive	43.17
54	F	43.45	18.00	IV	8	3.1400	5.4233	At diagnosis	–	CRD	35.37
56	F	45.19	10.00	III	7	7.7883	10.5417	No	128.50	Alive	128.50
57	F	47.00	3.50	I	1	8.1600	7.4840	No	84.00	Alive	84.00
58	F	47.69	5.00	I	0	–11.9350	–3.7350	No	79.30	Alive	79.30
59	M	54.00	4.50	I	4	6.9690	7.2920	No	25.00	Alive	25.00
62	F	64.06	3.50	I	0	6.5500	8.0783	No	45.97	Alive	45.97
63	F	65.32	6.00	II	4	2.6667	4.2567	No	54.73	Alive	54.73

DFS, disease-free survival; CRD, cancer-related death.

^aAccording to MacFarlane system.

a mean time of 15.67 months (6.9–35.4) after the diagnosis. The germline *TP53* mutation p.R337H was studied in 21 pediatric and 20 adult patients. In pediatric patients, it was identified in 13 (62%). In adults, two patients (10%) were carriers of *TP53* p.R337H germline mutations in heterozygous state (6).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from the frozen tumor fragments stored in liquid nitrogen using TRIzol (Invitrogen) reagent method. The RNA integrity and concentration were evaluated by agarose gel electrophoresis (1%) and spectrophotometry. cDNA was generated using the High Capacity cDNA Archive kit for RT (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed in the ABI PRISM 7000 Sequence Detector using TaqMan gene expression assays (Applied Biosystem). The assay IDs were *BUB1B*, Hs00177821_m1; *PINK1*, Hs00260868_m1; and *DLGAP5*, Hs007323_m1. A cycle threshold (C_T) value in the linear range of amplification was selected for each sample in triplicate and normalized to human beta glucuronidase (*GUSB*) endogenous control gene (4326320E). The differences of

ΔC_T (ΔC_T *DLGAP5*– ΔC_T *PINK1*) and (ΔC_T *BUB1B*– ΔC_T *PINK1*) were correlated with clinical and pathological parameters.

Statistical analyses

All statistical analyses were performed with the MedCalc Software (version 11.6.1.0; MedCalc Software BVBA, Brussels, Belgium). The adjusted cutoff values for (ΔC_T *DLGAP5*– ΔC_T *PINK1*) and (ΔC_T *BUB1B*– ΔC_T *PINK1*) were established by the receiver operating characteristic (ROC) curve method (see below). Univariate analysis was performed using Kaplan–Meyer curves and log-rank test statistics for comparison. A *P* value <0.05 was considered significant.

Results

DLGAP5–*PINK1* and disease-free survival

According to de Reyniès *et al.* (14), the subtraction of expression level of *DLGAP5* and *PINK1* (*DLGAP5*–*PINK1*) in ACTs could discriminate between malignant and benign tumors and was a predictor of disease-free survival. Accordingly, a sample is predicted to be

malignant if ($DLGAP5-PINK1$) < 6.95 . To find out whether similar results could be achieved in our cohort, we performed a Kaplan–Meyer survival analysis applying the same cutoff value to 23 adult patients with no evidence of residual disease or metastasis after surgery (absence of metastatic disease on preoperative evaluation and R0 surgical excision). All patients who remained disease free were followed for at least 24 months. The difference in disease-free survival between the two groups ($DLGAP5-PINK1 < 6.95$ and $DLGAP5-PINK1 \geq 6.95$) was significant (log-rank $P = 0.0315$). However, the performance of the molecular marker was apparently inferior to a Weiss score > 2 in prognosis prediction (log-rank $P = 0.0016$; Fig. 1A). We wondered whether applying a different cutoff value would improve the discriminative performance of the molecular marker. To select the best possible cutoff for our adult cohort, we applied the ROC curve analysis method. To build up the curve, we divided the 28 adult patients in two groups: i) unfavorable outcome (metastasis on diagnosis or recurrent/metastatic disease on follow-up; $n = 10$) and ii) favorable outcome (patients with no evidence of recurrence/metastasis after at least 24 months of follow-up; $n = 18$). The greatest area under the curve (AUC) value was obtained with a cutoff of ≤ 3.22 (AUC = 0.922, $P = 1.25 \times 10^{-12}$). This optimized cutoff value defined two groups of patients with significantly different disease-free survival (log-rank $P = 6.12 \times 10^{-6}$; Fig. 1B).

The next question was whether the molecular predictor was also discriminative in a pediatric cohort. To address this issue, we applied the adult cohort optimal cutoff value of ≤ 3.22 to a cohort of 21 pediatric patients with no evidence of residual disease after surgery. We observed a marginally significant difference in disease-free survival (log-rank $P = 0.0629$). Adjusting the cutoff value for the pediatric cohort did not improve its performance (log-rank $P = 0.0629$; Fig. 1D). As previously reported, in pediatric patients, a Weiss score > 2 is not a reliable predictor of malignancy (Fig. 1C).

BUB1B-PINK1 as a predictor of overall survival in ACCs

de Reyniès *et al.* (14) have demonstrated that the combined expression of $BUB1B-PINK1$ was the best predictor of overall survival in a cohort of histologically malignant ACTs. Accordingly, a sample was predicted to have a poor prognosis if ($BUB1B-PINK1$) < 6.32 . Applying the same cutoff values to 14 adult patients with histologically indeterminate or malignant ACTs (Weiss score ≥ 2), we could observe two groups with distinct survival time (log-rank $P = 0.037$). However, this molecular predictor was outperformed by the MacFarlane staging (log-rank $P = 0.007$; Fig. 2A). To select the best possible cutoff for ($BUB1B-PINK1$) in our adult cohort, we applied the ROC curve analysis method

Figure 1 $DLGAP5-PINK1$ as a predictor of disease-free survival. In 23 adult patients with no evidence of residual disease after surgery, a sample is predicted to present a poor outcome if the subtraction of C_t values of $DLGAP5-PINK1$ is < 3.22 . The molecular predictor of malignancy was at least as accurate as Weiss score (panels A and B). Among 21 pediatric patients, both the Weiss score and the molecular predictor could not discriminate groups with different outcomes, even after adjusting the cutoff for the best possible performance (panels C and D).

as described in the previous section. All patients that did not present an adverse outcome were followed for at least 60 months in this analysis. The greatest AUC value was obtained with a cutoff of ≤ 3.14 ($\text{AUC} = 0.907$, $P = 1.07 \times 10^{-6}$). This adjusted cutoff value leads to a better prediction of overall survival (log-rank $P = 4.56 \times 10^{-4}$; Fig. 2B). In the pediatric cohort with histologically indeterminate or malignant ACTs ($n = 19$), however, this molecular predictor was not associated with a statistically significant difference in overall survival, even after adjusting for the best possible cutoff value (log-rank $P = 0.146$; Fig. 2D). In the pediatric group, tumor stage remains the only significant parameter associated with overall survival (log-rank $P = 0.015$; Fig. 2C).

Discussion

ACC is a rare neoplasm with a dismal prognosis. Although the main prognostic factor is tumor stage, even localized disease (MacFarlane I and II) has a high relapse rate. The differential diagnosis between localized ACCs and adenomas is crucial and relies on a score of histological features (Weiss score), which has important drawbacks. To overcome the limitations of Weiss score, in the last few years, many authors have recently searched for molecular markers of malignancy. Among these, unsupervised hierarchical clustering of gene expression signatures has provided new insights into the identification of different groups of ACCs with distinct outcomes (14, 16). Giordano *et al.* (16) showed that cluster analysis revealed two subtypes of ACCs that reflected tumor proliferation, as measured by mitotic counts and cell cycle genes. de Reyniès *et al.* (14) also identified a subclassification of ACC based on the gene expression profile. Therefore, these two microarray-based assessment studies demonstrated that molecular signatures can robustly predict poor outcome with higher accuracy than morphological parameters (14, 16). However, these studies are difficult to perform and, consequently, unsuitable to the clinical practice. To overcome this limitation, de Reyniès *et al.* (14) identified three genes for which the combined expression values were highly associated with outcomes. According to their data, the difference in the expression values of *DLGAP5*–*PINK1* and *BUB1B*–*PINK1* were strong predictors of recurrence and overall survival, respectively, in adult ACT patients. *DLGAP5* and *BUB1B* are overexpressed in ACCs. The former is involved in stem cell pluripotency and carcinogenesis (17, 18) and the latter controls mitotic checkpoint and chromosomal segregation during mitosis (18, 19). *PINK1* gene, which is regulated by the *PTEN* gene and is involved with mitochondrial integrity, was found to be downregulated in ACTs (20). The authors suggested that due to its accuracy and simplicity, these molecular predictors would be relevant to clinical practice.

Figure 2 *BUB1B*–*PINK1* as a predictor of overall survival. Among 14 adult ACTs with a potentially malignant histology (Weiss ≥ 2), the difference between expression values of *BUB1B* and *PINK1* ≤ 3.14 was at least similar to MacFarlane staging in predicting a fatal outcome (panels A and B). Among 19 pediatric patients, however, only staging was associated to a fatal outcome.

Figure 3 *DLGAP5-PINK1* in 13 adult ACCs in which a R0 resection could be achieved. A cutoff value of 3.22 (dashed line) correctly classified all cases as 'recurrent' or 'non-recurrent'. Open circles: individual values; Filled squares: Mean values. Error bars: 95% CI.

In order to validate these findings, we studied the expression values of the same genes in ACTs from Brazilian patients. In the adult cohort, our results were quite similar as those reported (14), in which the molecular markers were strong predictors of recurrence and survival, with an accuracy at least comparable to clinical and histological parameters, if not better. It is noteworthy that five out of 13 cases of our adult cohort with a Weiss score ≥ 2 and R0 tumor resection presented a relapse on follow-up. The molecular predictor (*DLGAP5-PINK1*) ≤ 3.22 correctly identified all cases as 'non-recurrent' and 'recurrent' (Fig. 3). In spite of the limited number of cases, these data are promising, suggesting that the molecular marker goes beyond clinical and histological information in prognosis prediction.

The differences in cutoff values of *DLGAP5-PINK1* and *BUB1B-PINK1* between our study and those reported by de Reyniès *et al.* (14) may be related to methodological issues. Nevertheless, external validation is important when there is inter-tumor heterogeneity; notably, both studies corroborate the reliability of these molecular markers in the clinical evaluation of adult ACT. In addition, we first employed these new molecular markers to predict outcome in a cohort of pediatric ACTs diagnosed in Brazilian patients. In this group of patients, these molecular markers were not good discriminators of outcome, although a marginally significant trend could be observed in disease-free survival curves (Fig. 1D).

In conclusion, we validated the prognostic value of the combined expression of *BUB1B*, *DLGAP5*, and *PINK1* genes in a different group of adult ACTs and reinforced its potential applicability to clinical practice. For pediatric patients, according to our data, these molecular markers are still lacking sufficient discriminatory efficiency and other markers need to be identified.

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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