IGF2 and IGF1R in pediatric adrenocortical tumors: roles in metastasis and steroidogenesis

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Abstract

Deregulation of the IGF system observed in human tumors indicates a role in malignant cell transformation and in tumor cell proliferation. Although overexpression of the *IGF2* and *IGF1R* genes was described in adrenocortical tumors (ACTs), few studies reported their profiles in pediatric ACTs. In this study, the *IGF2* and *IGF1R* expression was evaluated by RT-qPCR according to the patient's clinical/pathological features in 60 pediatric ACT samples, and IGF1R protein was investigated in 45 samples by immunohistochemistry (IHC). Whole transcriptome and functional assays were conducted after IGF1R inhibition with OSI-906 in NCI-H295A cell line. Significant *IGF2* overexpression was found in tumor samples when compared with non-neoplastic samples ($P < 0.001$), significantly higher levels of *IGF1R* in patients with relapse/metastasis ($P = 0.031$) and moderate/strong IGF1R immunostaining in 62.2% of ACTs, but no other relationship with patient survival and clinical/pathological features was observed. OSI-906 treatment downregulated genes associated with MAPK activity, induced limited reduction of cell viability and increased the apoptosis rate. After 24 h, the treatment also decreased the expression of genes related to the steroid biosynthetic process, the protein levels of the steroidogenic acute regulatory protein (STAR), and androgen secretion in cell medium, supporting the role of IGF1R in steroidogenesis of adrenocortical carcinoma cells. Our data showed that the *IGF1R* overexpression could be indicative of aggressive ACTs in children. However, *in vitro* treatments with high concentrations of OSI-906 ($> 1 \mu\text{M}$) showed limited reduction of cell viability, suggesting that OSI-906 alone could not be a suitable therapy to abolish carcinoma cell growth.

Key Words

- ▶ *IGF2*
- ▶ *IGF1R*
- ▶ childhood
- ▶ adrenocortical tumor
- ▶ OSI-906

Endocrine-Related Cancer
(2016) 23, 481–493

Introduction

Pediatric adrenocortical carcinoma (ACC) is a rare neoplasia with a worldwide incidence estimated at 0.2–0.3 cases/million children under 15 years per year (Else et al. 2014). The incidence in Southern of Brazil is 10–15 higher than the worldwide rate, which is related to the inherited germline *TP53* mutation (*p.R337H*) (Ribeiro & Figueiredo 2004). Some of prognostic factors include older age, mitotic rate, tumor weight, tumor size, and presence of metastasis (Klein et al. 2011). Complete surgical resection remains the only treatment to achieve cure and long-term survival, which is less than 30% in patients with advanced stages of the disease (Fassnacht et al. 2011, Lorea et al. 2012). Adjuvant mitotane, chemotherapy and/or radiotherapy have been often recommended in order to reduce local recurrence. For palliative cases, the arterial chemoembolization, radiotherapy and radiofrequency ablation should also be considered (Berruti et al. 2012, Fassnacht et al. 2012, Else et al. 2014).

Among molecular markers, the overexpression of insulin growth factor 2 (*IGF2*) has been commonly found in pediatric tumors (Wilkin 2000, Lerario et al. 2014). Additionally, few studies have evaluated the insulin growth factor 1 receptor (*IGF1R*) gene expression (West et al. 2007, Almeida et al. 2008). The IGF signaling is activated by IGF1, IGF2, and/or insulin binding to the IGF1R, which autophosphorylates and begins downstream cascades such as PI3K and MAPK, promoting cell proliferation and differentiation and exerting anti-apoptosis effects and angiogenesis (Riedemann & Macaulay 2006, Pollak 2012). Although adrenal tumorigenesis involves several genetic abnormalities, the *IGF2* overexpression seems to occur at an earlier stage of tumor formation, but it is unable to cause tumor formation alone (Assié et al. 2014, Pinto et al. 2015).

Evidences of the IGF pathway role in malignant cell transformation and proliferation have conducted to the study of IGF1R target-drugs. Among them, OSI-906 (Linsitinib) is a potent, oral, and selective inhibitor of the IGF1R and insulin receptor (IR) autophosphorylation, reducing cell proliferation in different types of cancer cell lines (Mulvihill et al. 2009). Recently, a large phase III trial in adults with advanced ACC demonstrated no differences in overall survival between patients treated with OSI-906 and the placebo group (Fassnacht et al. 2015, Kirschner 2015). Therefore, it remains to be established the real role of IGF1R in ACT and whether anti-IGF1R therapy alone

or combined with other drugs is efficient as a preferred treatment.

In this study, we analyzed *IGF1R* and *IGF2* gene expression profiles according to the clinical and pathological features of adrenocortical tumors in a large series of pediatric patients' samples and investigated the effect of IGF1R inhibition by OSI-906 in the ACC cell line NCI-H295A as well as the altered downstream signaling pathways.

Subjects, material, and methods

Patients

A total of 60 pediatric adrenocortical tumor samples were obtained from the University Hospital, Ribeirao Preto Medical School, University of Sao Paulo, and Boldrini Children Center, Campinas. All patients underwent clinical and hormonal evaluation by biochemical and imaging investigation. Abdominal and chest CT and bone scintigraphy were conducted for metastasis detection at diagnosis and during follow-up. The disease stage at diagnosis was based on modified Sandrini's classification of childhood ACTs (Michalkiewicz et al. 2004). Ten non-neoplastic adrenal samples were used as control, which were collected during nephrectomy due to Wilms' tumor, before chemotherapy and from children without Beckwith–Wiedemann syndrome. The study was approved by the local Ethics Committees (protocol number: 8380/2010), and a signed statement of informed consent was obtained from the children's parents.

The group of patients diagnosed with ACT consisted of 46 girls and 14 boys with a mean age at diagnosis of 40.5 months (range 5–187 months). Three patients had nonsecreting tumors, while 57 had hormone-secreting tumors (37 androgen, 18 mixed cortisol/androgen, and 2 cortisol). Thirty-five patients were classified as stage I, 10 as stage II, 8 as stage III, and 7 as stage IV. The germline *TP53* p.R337H mutation was evaluated by direct genomic DNA sequencing and was detected in 52/60 (86.6%) patients. In most tumors, DNA was sequenced and the loss of heterozygosity (LOH) at the 17q locus was confirmed. The analysis of the entire coding and boundary regions of the *TP53* gene revealed the absence of other mutations. Median follow-up was 68.3 months (range: 8–168 months). Twenty patients (33.3%) presented metastasis at diagnosis ($n=7$) or relapsed ($n=13$). The clinical and pathological features of these patients were described previously (Leal et al. 2011, Lorea et al. 2012, Gomes et al. 2014).

Real-time PCR (qPCR)

Tumor fragments were collected during surgical resection, frozen in liquid nitrogen, microdissected, and revised by a pathologist. Total RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's instructions. The cDNA was generated from 1 µg total RNA using the High Capacity Kit (Applied Biosystems). The human genes *IGF2* (Hs01005963_m1), *IGF1R* (Hs00609566_m1), *MAPK1* (Hs01046830_m1), *MAPK3* (Hs00385075_m1), and *PIK3R5* (Hs01046353_m1) were amplified by qRT-PCR using TaqMan gene assays and the ABI 7500 Real Time PCR System (Applied Biosystems). All samples were analyzed in triplicate and normalized to the endogenous reference human genes *ACTB* and *GUSB* (Applied Biosystems) as described previously (Leal et al. 2011, Leite et al. 2014). The relative expression was determined by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), and the median gene expression values of all non-neoplastic adrenal tissues was used as reference and defined as 1 for analysis of tumor samples. For *in vitro* assays, samples were normalized to the *GUSB* gene, and untreated cells were used as reference samples.

Immunohistochemistry

Immunohistochemistry for IGF1R was performed by the avidin–biotin peroxidase complex (ABC) method (Novocastra, Newcastle-upon-Tyne, UK) in 45 tumor samples from the 60 patients evaluated in this study. Fifteen samples were excluded because they presented few or no tumor representative area anymore. A small group of 21 tumors was also evaluated for IGF2. The primary antibody was applied for overnight incubation (mouse monoclonal anti-IGF-1R, 1:300; Biocare Medical – CM 414 A. C.; Concord, CA, USA; mouse monoclonal anti-IGF2, 1:50; Santa Cruz Biotechnology, sc-74119) and a biotinylated secondary antibody was incubated. The visualization was performed with streptavidin peroxidase followed by diaminobenzidine coloring (Gibco) and Harris' hematoxylin counterstaining. The positive control was a breast carcinoma sample (IGF1R) and placenta (IGF2) and the negative control was obtained by replacing the primary antibody with PBS 1X.

The immunohistochemistry analysis classified the samples as negative (no/weak staining) or positive (moderate/strong staining) according to the intensity of IGF1R/IGF2 staining. Regarding the localization, the samples were grouped into cytoplasmic, cytoplasmic/membrane, or nuclear staining.

Cell line and reagents

The human adrenocortical carcinoma cell line NCI-H295A was cultivated in RPMI medium as described previously (Gomes et al. 2014). Cell line authentication was conducted by examining CSF1PO, D13S317, D16S539, D5S818, D7S820, THO1, TPOX, vWA, and AMEL polymorphic loci by short-tandem repeat (STR) profiling. To avoid genetic drift or selection of variant subclones, all experiments were performed under standard cell culture conditions in an incubator at 37°C in a humidified atmosphere of 5% CO₂ and cells were used at low passages (<10).

The OSI-906 inhibitor was acquired from Selleck Chemicals (LLC, Houston, TX, USA), dissolved in dimethyl sulfoxide (DMSO) at stock concentration of 10mM, and stored at –20°C. Control groups were prepared for all experiments using cells grown in medium with DMSO only.

Whole-transcriptome analysis

After NCI-H295A treatment with OSI-906 (2 µM) for 6 and 24 h, total cellular RNA was extracted using Trizol Reagent (Invitrogen) and stored in DEPC-treated water at –80°C, and the quantity and quality of samples was evaluated with an ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The mRNA library was constructed using 200 ng total RNA and investigated with the Whole Human Genome Microarray Kit, 4x44K (Agilent Technologies) to determine the gene expression profiles. Spot images were processed by Feature Extraction Software v10.7.3.1 (Agilent Technologies). All steps of quality evaluation, normalization, background correction, and statistical analysis were performed using R statistical language (Gentleman et al. 2004, R Development Core Team 2014) and the Bioconductor package Agi4x44PreProcess: PreProcessing of Agilent 4x44 array data (Lopez-romero 2012). After background correction, the samples were quantile normalized and the expression of the genes was obtained using a linear model fitted to each gene, so that the fold change between conditions and its associated errors could be estimated. Differentially expressed genes were obtained from those with lowest *P*-value and extreme values of fold-change. To annotate the differentially expressed genes (DEGs), EnrichR tool was used for gene ontology (GO) and KEGG pathway analysis with criterion of *P*-value < 0.05 and at least three genes per process (Chen et al. 2013).

Cell viability assay

The resazurin reduction method was used to investigate cellular metabolic activity after treatment with OSI-906 (O'Brien *et al.* 2000). For the assay, the cells were treated with OSI-906 inhibitor at different concentrations (0.125–3 μ M) for 24, 48, or 72 h. After the treatment period, resazurin (Sigma-Aldrich) was added and the plates were incubated for 4 h at 37°C, 5% CO₂.

Absorbance at 570 nm wavelength was then read with a reference wavelength of 595 nm using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories). The effects of OSI-906 in the cell viability were reported as mean $\pm$ s.d. of at least three independent experiments performed in triplicate. The EC₅₀ values were calculated using CalcuSyn software (Biosoft, Ferguson, MO, USA) (Chou & Talalay 1984).

Apoptosis assay

Apoptotic cell death was determined by labeling with annexin V fluorescein isothiocyanate (BD Biosciences Pharmingen, San Jose, CA, USA) and propidium iodide (PI) staining. Briefly, after 72 h of OSI-906 treatment, 1.5×10^5 cells were trypsinized and centrifuged at 112 *g* for 5 min at 4°C, washed with ice-cold PBS 1X, and then resuspended in 350 μ L annexin V binding buffer (BD Biosciences Pharmingen, San Jose, CA, USA). Cells were stained with 5 μ L annexin V and 50 μ L PI 50 μ mol/L and incubated at room temperature in the dark. The samples were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences Pharmingen, San Jose, CA, USA).

Cell lysis and western blot analysis

After treatment with OSI-906 (2 μ M) for 3, 6, or 24 h, cells were lysed in RIPA buffer (Sigma-Aldrich) in the presence of protease and phosphatase inhibitors. Equal amounts of whole-cell lysates were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) followed by transfer to nitrocellulose membranes (GE Healthcare), which were incubated in Tris-buffered saline and 0.1% Tween-20 containing 5% (w/v) dried nonfat milk for 1 h at room temperature and probed with appropriately diluted primary antibodies overnight at 4°C. Blots were incubated with biotin-labeled horseradish peroxidase-conjugated species-specific secondary antibodies (1:10,000; GE Healthcare) followed by chemiluminescence detection using the ECL Western blotting Analysis System Kit (Amersham GE Healthcare) and ChemiDoc System (Bio-Rad Laboratories).

Rabbit monoclonal antibodies against phospho- and total-ERK1/2 were obtained from Cell Signaling (respectively, #9101 and #9102; both 1:800). A rabbit polyclonal antibody against STAR was purchased from Santa Cruz Biotechnology (1:200; sc-25806) and a mouse monoclonal antibody against GAPDH from Santa Cruz Biotechnology was used as loading control (1:1000; sc-47724).

Figure 1

(A) *IGF2* mRNA overexpression in adrenocortical tumor samples when compared with non-neoplastic adrenal samples. (B) Significant *IGF1R* overexpression in samples from patients with relapse or metastasis compared with patients with complete remission. Real-time PCR analysis was performed with samples in triplicate, which were normalized against the *ACTB* and *GUSB* genes. Statistics: Mann-Whitney test; FC, fold-change value. The empty and the full circles represent values above and below the 25 and 75 percentiles for the relative expression the *IGF2* gene in non-neoplastic adrenal and tumor samples.

Figure 2

Representative images of IGF1R protein expression in adrenocortical tumor tissues, detected by immunohistochemistry. (A) Non-neoplastic adrenal tissue adjacent to the tumor with negative staining for IGF1R. (B) Adrenocortical tumor cells with intense cytoplasmic staining surrounded by negative areas. (C) Tumor cells with both cytoplasmic and membranous staining. (D) Focal area of tumor cells with intense nuclear staining. Original magnification $\times 400$. A full color version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0426>.

Hormone measurements

After 24 h of OSI-906 treatment ($2\mu\text{M}$), the supernatant medium was collected from each well before cell lysis for RNA or protein extraction and stored at -80°C until hormone quantification. Dehydroepiandrosterone sulfate (DHEAS), D4-androstenedione (D4), and testosterone concentrations were determined by radioimmunoassay (RIA) as described previously (Moreira & Elias 1992). All measurement of hormones from the NCI-H295A medium was conducted in triplicate and the mean was normalized to the cell viability effects in the same treatment conditions.

Evaluable data from circulating hormone concentrations (Cortisol, DHEAS, D4, Testosterone) of patient before tumor treatment was used for correlations analysis.

Statistical analyses

The expression of *IGF2* and *IGF1R* genes according to following variables: age ($<$ versus ≥ 4 years), tumor weight ($<$ versus $\geq 100\text{g}$), tumor size ($<$ versus $\geq 200\text{cm}^3$), *TP53* p.R377H mutation (positive vs negative), and disease stage was compared by the Mann–Whitney test. Differences between the expression values are reported as fold change (FC) by dividing the median expression values for each variable analyzed. Event-free survival (EFS) analysis (with relapse and/or death due to any cause being considered as unfavorable events) was carried out based on Kaplan–Meier curves, using the gene expression median values

from ACTs as the cut-off point for *IGF2* and *IGF1R*. The curves for different groups were compared by the log-rank test. Correlations were determined using the Spearman correlation coefficient (ρ).

Data of the functional assays such as cell viability and apoptosis, as well as the gene expression after OSI-906 treatment, were evaluated by one-way ANOVA with the Bonferroni post-test. Moreover, differences of hormone concentrations in the cell medium were analyzed by the t-test for equality of means. All analyses were carried out using the IBM SPSS Statistics software version 20 (SPSS), with the level of significance set at 0.05.

Results

IGF2 gene is overexpressed in adrenocortical tumors

A significant overexpression of *IGF2* was detected in ACT samples when compared with non-neoplastic adrenal tissue, (15.9-fold, $P < 0.001$) (Fig. 1A); however, *IGF2* overexpression was not related to relapse or metastasis in the patients ($P = 0.243$), neither with clinical–pathological features nor with 5-year EFS ($P = 0.753$).

IGF1R gene expression is higher in patients with relapse and metastasis

Although *IGF1R* gene expression was very similar for non-neoplastic adrenal samples and ACTs ($P = 0.822$), we observed significant overexpression of *IGF1R* in

Figure 3

Main biological processes associated with up- and downregulated differentially expressed genes (DEGs) after inhibition of IGF1R by treatment with OSI-906 in NCI-H295A cells. (A) Downregulated DEGs after 6h of treatment were associated with positive regulation/activation of MAPK pathway and cell motility. (B) Downregulated DEGs after 24h of treatment, enriched in lipid and hormone synthesis/metabolism, response to external stimuli and lipid metabolic process. (A and B) Upregulated DEGs were enriched in activation/positive regulation of caspases and cell cycle regulation/arrest at both treatment times.

patients with tumor relapse or metastasis when compared with the group of patients with complete remission (2.7-fold, $P=0.031$) (Fig. 1B). However, *IGF1R* overexpression ($1\times$ or $2\times$ >median) was not indicative of significant differences in the EFS of patients with ACT ($58.7\pm 13.0\%$

vs $80.6\pm 6.2\%$, $2\times$ >median; $P=0.103$). Interestingly, we found significant positive correlation between *IGF1R* gene expression and DHEAS concentrations in patient's serum ($\rho=0.663$; $P=0.02$).

Transcripts do not correlate with protein expression

The IGF1R protein expression evaluated by IHC did not agree with the gene expression profiles observed by qRT-PCR. Positive immunoreactivity was found in 32/45 (71.1%) of the ACT samples, with 16 cases (35.5%) classified as strong, 12 (26.7%) as moderate, and 4 (8.9%) as weak. The remaining 13 samples (28.9%) as well as the non-neoplastic tissue adjacent to the tumor presented no IGF1R staining (Fig. 2). Moderate or strong immunostaining were considered as IGF1R positive, while samples with weak or absent staining were classified as IGF1R negative. The median of *IGF1R* mRNA expression was 0.58 for IHC-positive samples (mean: 1.46, range: 0.10–8.26) and 0.55 for IHC-negative samples (mean: 1.23, range: 0.17–4.88) ($P=0.933$). Cytoplasmic staining was observed in 26 tumor samples, cytoplasmic and focal membrane staining was present in 4 samples, and focal nuclear staining was detected in 2 cases. There was no significant difference in 5-year EFS between patients with moderate/strong and negative/weak IGF1R immunoreactivity ($P=0.613$) or with pure cytoplasmic versus membranous/nuclear staining ($P=0.726$).

Additionally, the IHC for IGF2 showed no correlation with transcripts, being 43% of negative cases, 43% with weak staining, 10% with moderate, and 5% with strong staining. The positive samples presented predominantly cytoplasmic staining (data not shown), and no significant differences were found according to the clinical and pathological features.

Identification of differentially expressed genes after treatment of adrenocortical carcinoma cells with OSI-906

In order to investigate the role of IGF1R in adrenocortical carcinoma cells (NCI-H295A), the global gene expression by microarray analysis was performed after functional blockage of IGF1R with OSI-906. After normalization, the 2000 most differentially expressed genes (DEGs) at two time points during treatment (6 and 24h) were selected for further analysis. Biological processes (gene ontology (GO)) and pathway mappings (KEGG) were evaluated for the 200 most upregulated and the 200 most downregulated genes for each treatment time.

Table 1 KEGG Pathways enrichment of downregulated DEGs after 24 h of OSI-906 treatment.

Term	Gene count	P-value	Z-score	Combined score	Genes
HSA00140 – C21 steroid hormone metabolism	5	9.65×10^{-7}	-1.62	15.58	<i>HSD3B2; HSD3B1; CYP21A2; CYP11B1; CYP11B2</i>
HSA00330 – arginine and proline metabolism	4	0.001	-1.88	5.84	<i>CKB; CKMT2; EPRS; OAT</i>
HSA00150 – androgen and estrogen metabolism	4	0.006	-2.00	4.10	<i>CYP11B1; CYP11B2; HSD3B2; HSD3B1</i>
HSA03320 – PPAR signaling pathway	4	0.013	-1.72	2.92	<i>APOA1; ACSL6; FADS2; FABP6</i>
HSA01510 – neurodegenerative diseases	3	0.014	-1.57	2.66	<i>UCHL1; NEFH; PRNP</i>
HSA01040 – polyunsaturated fatty acid biosynthesis	2	0.016	-0.50	0.86	<i>FADS2; ELOVL5</i>
HSA00642 – ethylbenzene degradation	2	0.018	-0.61	1.04	<i>ESCO2; DHRS2</i>
HSA00624 – 1 and 2 methylnaphthalene degradation	2	0.044	-1.00	0.96	<i>ESCO2; DHRS2</i>

The results demonstrated that upregulated DEGs were significantly enriched in the GO factors involved in biological processes such as cytoskeleton organization and NFκB cascade, while the downregulated DEGs were enriched in hormone metabolic/biosynthetic processes and MAPK activity.

We next evaluated whether treatment with OSI-906 could also impair time-dependent changes in the biological processes. At both times, the upregulated DEGs were enriched in GO terms such as activation/positive regulation of caspases and cell cycle regulation/arrest, while one KEGG pathway was identified only at 6 h of

Figure 4

Effects of OSI-906 in NCI-H295A cells. (A) Reduced *MAPK1* gene expression after 6 h of treatment with 2 and 4 μM OSI-906, reinforcing the enrichment of the downregulated DEGs at 6 h of treatment; (B) Significant reduction in *PI3K* gene expression 24 h after treatment; (C) Trend of reduction and increase in *MAPK3* gene expression after 6 and 24 h of treatment, respectively (NS, non-significant). (D) The western blotting analysis reinforced the qRT-PCR, showing reduced expression of phosphorylated and total ERK1/2, which reached the lowest level after 6 h of treatment. (E) Significant and stable reduction in cell viability after treatment with OSI-906. At 24 h, the reduction was significant with doses 1–3 μM, while after 48 and 72 h, all doses induced significant decrease in cell viability. Time-dependent reduction was observed since at the lowest dose (*significant difference compared with the control DMSO; #time-dependent significant difference). (F) Significant increase in apoptotic cells after 72 h of treatment with OSI-906. Bars represent the means of three independent experiments with three replicates per experiment. Error bars indicate the s.d. Statistics: One Way ANOVA with Bonferroni post-test.

Figure 5

Reduction of hormone secretion by NCI-H295A cells treated with OSI-906 for 24h. (A) Dehydroepiandrosterone sulfate (DHEAS). (B) Testosterone. (C) D4-androstenedione (D4). (D) Reduction in STAR protein levels after 24h of treatment with OSI-906, confirming the inhibition of the general pathway of hormone synthesis. Statistics: T-test for equality of means; NS, nonsignificant.

treatment (HSA04115 – P53 signaling pathway; $P=0.03$). On the other hand, the group of downregulated DEGs were associated with positive regulation/activation of the MAPK pathway and cell motility at 6h (Fig. 3A) and, significantly enriched in lipid and hormone synthesis/metabolism, response to external stimuli and lipid metabolic processes (Fig. 3B) at 24h. The downregulated DEGs were also enriched in KEGG pathways related to hormone metabolism (Table 1), supporting the findings of the biological processes enrichment. Moreover, differential expression of specific genes such as higher expression of *DEPTOR* (FC=1.4) and *IRS2* (FC=1.4) at 24h, as well as reduction in *PPARGC1B* (FC=-1.6) at 24h and *PIK3C2G* (FC=-1.4) at 6h suggests inactivation of mTOR signaling downstream IGF1R.

Confirming the microarray analysis by qRT-PCR, the cells treated with OSI-906 showed significant reduction in *MAPK1* at 6h and *PI3K* at both times. *MAPK3* expression was slightly lower at 6h and higher at 24h (not significant) (Fig. 4A and C). Moreover, the treated cells showed reduction in phosphorylated and total ERK1/2 proteins when compared with untreated cells (Fig. 4D).

OSI-906 reduces tumor cell viability and induces apoptosis

Treatments with 1, 2, and 3 μ M of OSI-906 for 24h decreased cell viability by 13, 15 and 18%, respectively, while at 48 and 72h, the reduction was significant with all doses of treatment (0.125–3 μ M) ($P<0.001$). However, limited reductions were observed at concentrations beyond 0.5 μ M, which were characterized by a plateau in the graph of cell viability (Fig. 4E). The most effective reduction was observed at 72h with 1 μ M (40%). The time-dependent effect was confirmed with distinct EC_{50} ($>20 \pm 15.2 \mu$ M for 24h; $2.64 \pm 0.23 \mu$ M for 48h; $1.99 \pm 0.78 \mu$ M for 72h). We also observed a significant dose-dependent increase in cell apoptosis rate at 72h, reaching 31% of apoptotic cells with 1 μ M of OSI-906 (CI: 62.4–79.3; $P<0.001$) (Fig. 4F).

Treatment with OSI-906 reduces hormone biosynthesis in adrenocortical carcinoma cells

To validate the microarray findings concerning the reduction in genes associated with hormone synthesis

and lipid metabolism, we investigated the effects of OSI-906 on cellular hormone production after 24 h. All steroid hormones were reduced, but it was significant only to testosterone ($P=0.02$) (Fig. 5A and C). Interestingly, STAR protein expression decreased 55% at 24 h (Fig. 5D).

Discussion

The characteristics of our samples regarding clinical data such as age, sex, tumor stage, clinical symptoms, survival, and prognostic factors were similar to those described by the International Pediatric Adrenocortical Tumor Registry (IPACTR) (Michalkiewicz et al. 2004).

One hallmark of pediatric ACT in Brazil is the *TP53* p.R337H germline mutation, which disrupts protein tetramer formation and reduces the p53 activity in higher pH levels (Wasserman et al. 2012). Interestingly, in response to cellular stress such as oncogene activation, the p53 can shutdown the IGF pathway, reducing IGF2 and IGF1R expression and the receptor tyrosine phosphorylation (Sampaoli et al. 2012). Similar to other Brazilian childhood ACT series (Sandrini et al. 2005, Custódio et al. 2013), we observed high frequency of *TP53* p.R337H mutation in tumor samples, but no significant differences in *IGF2* or *IGF1R* expression profiles between samples with or without the mutation.

In vitro studies have shown that apoptosis and inhibition of ACC cells growth after ionizing radiation is dependent of p53 protein stabilization (Sampaoli et al. 2012), and *TP53* somatic mutations confer resistance for anti-IGF1R therapy in colorectal carcinoma cells (Wang et al. 2013). Interestingly, NCI-H295 lacks *TP53* p.R337H, but presents other mutations affecting p53 functions (Sampaoli et al. 2012, Leal et al. 2015), which also could be related to reduced effectiveness of therapies, such as anti-IGF1R.

The role of the IGF system in the development and growth of adrenal cortex is well known, with high levels of IGF2 detected in the adrenal glands and serum during the fetal stage, followed by a strong decline during the postnatal period. However, overexpression of IGF2 has been associated with a higher risk of ACT recurrence in adults (Boulle et al. 1998, Fottner et al. 2004). In children, we observed that *IGF2* overexpression was not associated with tumor recurrence or disease poor outcome, which is in agreement with a few studies reporting *IGF2* higher expression in pediatric ACT (Wilkin 2000, West et al. 2007, Almeida et al. 2008).

Among the four receptors of the IGF pathway described in mammals, the tyrosine kinase receptor type 1 (IGF1R) originates signals that facilitate cell transformation by other agents in different types of tumors (Wang & Sun 2002). In agreement with its pivotal role in cell growth and homeostasis, the overexpression of *IGF1R* in several human cancers is not surprising and suggests the involvement of IGF1R in tumor growth and progression (Maki 2010). In this study, *IGF1R* expression was quite similar for non-neoplastic and tumor samples, but patients with metastasis or relapse showed significantly higher *IGF1R* expression levels. Similar to our findings, Almeida and coworkers (2008) observed *IGF1R* overexpression in adrenocortical carcinomas and significant association with higher risk of metastasis in a study with 23 pediatric ACT samples. Herein, all control samples consisted of non-neoplastic adrenal tissue with both cortical and medullary cells, which somehow could be a potential bias for the analysis.

The correlation between correspondent IGF1R and IGF2 protein expression and their transcripts were not significant in the evaluated ACT samples. Other studies on cancer have revealed that the lack of correlation between mRNA and protein levels can be attributed to mRNA translational silencing, cleavage, or alternative splicing (Dzadziesko et al. 2010, Mountzios et al. 2013). In the IGF1R context, alternative mRNA splicing can lead to distinct protein degradation rates in cancer cells (Mountzios et al. 2013) while the frequently expressed isoform (alpha) of IGF1R is not directly associated with global *IGF1R* gene expression (Pollak 2012). Interestingly, reports have demonstrated the impact of IGF1R protein localization on the biological behavior and prognosis of human breast cancer and in clear renal cancer cells (Aleksic et al. 2010, Tamimi et al. 2011). The ACT samples presented more IGF1R pure cytoplasmic than mixed cytoplasmic/membranous/nucleus staining, but no significant differences were observed regarding patients' survival, suggesting that IGF1R localization has no prognostic relevance for pediatric ACTs.

Evidence of IGF signaling in malignant cell transformation and tumor cell proliferation has encouraged the development of many IGF1R target-drugs. Among them, OSI-906 is known to reduce tumor cells proliferation because of its selective effect on both IGF1R and IR (Mulvihill et al. 2009). According to the global gene expression analysis of NCI-H295A cells, the inhibition of IGF1R with OSI-906 seems to upregulate caspases activity and to induce cell cycle arrest. Gene array analysis conducted on a broad panel of colorectal cancer cell lines

revealed that OSI-906-sensitive cells present upregulation of the P53 pathway, while resistant cells present MAPK pathway upregulation (Pitts et al. 2010). At 6 h of OSI-906 treatment, we observed the upregulation of P53 signaling pathway genes and reduction of genes associated with MAPK pathway activation, suggesting a sensitive profile of the NCI-H295A cell line. In fact, it was obtained a significant dose-dependent increasing of apoptosis rate (31%), but cell viability was limited to 40% even after high doses of OSI-906. Since resistant colorectal cancer cells present upregulation of the WNT pathway (Pitts et al. 2010), the mild decrease of cell viability in NCI-H295A cells could be related to the constitutive activation of Wnt/ β -catenin signaling due to S45P mutation in this cell line (Tadjine et al. 2008).

Reduction of cell viability after treatment with OSI-906 has been frequently reported in different types of cancer cells. In this study, we used doses between 0.125 and 3 μ M, which are comparable with other preclinical studies and are also within the range of human maximal plasma concentrations ($C_{\max}$ from 1.705 to 3.110 μ M) after oral administration of OSI-906 (150 mg of OSI-906 twice daily) (Fassnacht et al. 2015, Puzanov et al. 2015). Sensitive cells, including the ACC cell line NCI-H295R, usually present $EC_{50} < 1 \mu$ M at 72 h (Buck et al. 2010, Zhao et al. 2012, Janku et al. 2013). Surprisingly, we found higher EC_{50} values ($>20 \mu$ M at 24 h, 2.6 μ M at 48 h and 2.0 μ M at 72 h) which, together with the restoration of ERK-1/2 expression after 24 h of treatment, suggest a resistant profile for NCI-H295A after long periods of treatment (Zinn et al. 2013). Since OSI-906 inhibits kinase activities from both IR and IGF1R, we ruled out a resistance mechanism through compensatory IR signaling (Buck et al. 2010). However, new functions for IGF1R have been described (Boucher et al. 2010, Janku et al. 2013), which can explain the significant increase in apoptosis with only a mild decrease in NCI-H295A viability observed after OSI-906 treatment. Janku and coworkers (2013) showed that IGF1R is able to keep intracellular glucose levels, supporting tumor cell survival independent of their kinase activity. Moreover, IGF1R interacts with other receptor tyrosine kinases such as epidermal growth factor receptors (EGFRs), vascular endothelial growth factor receptor (VEGFR), mesenchymal–epithelial transition factor (MET), platelet-derived growth factor receptor (PDGFR), estrogen receptors (ER), and others, which are frequently found upregulated in cancer cells and may play a role in resistance to therapies anti-IGF1R. The activation of common downstream effectors through other receptors has provided new approaches regarding

cotargeting strategies for anticancer therapies (Singh et al. 2014, Brahmkhatri et al. 2015).

In addition, OSI-906 reduced *PIK3C2G* (catalytic subunit type 2 Gamma of PI3K) gene expression in the microarray analysis and the *PI3K* gene by qPCR. The PI3 kinases family transduces signals from various growth factors such as IGF-IGF1R, which activates downstream mTOR (mammalian target of rapamycin) signals (Liu et al. 2009). Activation of mTOR is induced by two complexes (TSC1 and TSC2), driving cancer cells growth and proliferation (Advani 2010). After OSI-906 treatment, the cells also expressed higher levels of *DEPTOR* gene, a negative regulator of mTORC1 and mTORC2, as well as lower expression of *PPARGC1B* and increased expression of *IRS2*, which, together with *PI3K* reduction, suggest the inactivation of mTOR pathway (Laplane & Sabatini 2009, Brouwer-Visser & Huang 2015).

Adrenocortical tumors are frequently characterized by hormonal secretion, inducing clinical symptoms of the disease or revealing tumor recurrence during follow-up (Göncü et al. 2014). As expected, most of the children evaluated presented pure androgen or mixed (androgen and cortisol) secreting tumors, while only two patients presented cortisol secretion alone. *In vitro* studies have shown that IGF1/IGF1R stimulates hormone synthesis in different steroidogenic cells by inducing the expression of steroidogenic genes and the steroidogenic acute regulatory (STAR) protein through MAPK/ERK signaling (Ramanjaneya et al. 2011). In agreement, we found significant positive correlation between *IGF1R* gene expression and DHEAS levels in diagnosis patient's serum. Moreover, cells treated with OSI-906 presented lower expression of genes related to hormone synthesis/metabolism and STAR protein impairment of 55%, which is considered the first key mediator of steroidogenesis (Samandari et al. 2007).

To a lesser extent, only testosterone concentration was significantly reduced after OSI-906 treatment, suggesting that steroidogenesis impairment STARTed with lower STAR protein expression could not be sufficient to exert downstream effects in the synthesis of all steroid hormones at 24 h.

In summary, *IGF2* gene overexpression in a relatively large series of children diagnosed with ACT was not related to any clinical or biological features analyzed here, while *IGF1R* gene expression was significantly higher in children who presented tumor relapse and metastasis, which was not true for the IGF1R protein expression analyzed by IHC. *In vitro* blockage of IGF1R signaling downregulated MAPK activity, causing reduction in cellular viability and increase in apoptosis rate in a dose-dependent manner. In addition,

OSI-906 decreased the expression of genes related to the steroid biosynthetic process and impaired the expression of STAR, a key steroidogenic enzyme, events that were followed by the reduction in testosterone production. These findings suggest that IGF1R could have a role in adrenocortical cancer; however, its inhibition by OSI-906 in adrenocortical tumor cells seems to promote only a mild antitumoral effect in a similar way as observed in clinical trials. Thus, new studies which elucidate the mechanisms of resistance to OSI-906 as well as new therapy schedules, such as drugs combination, would be important for a clinical application of IGF1R as a therapeutic target in childhood adrenocortical cancer.

Declaration of interest

All authors declare that they had no conflict of interest that could be perceived to impair the impartiality of the research reported.

Funding

This work was supported by the Public Research Agencies: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Grant number: 2010/07020-9; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Apoio ao Ensino, Pesquisa e Assistência do Hospital das Clínicas – FMRP/USP (FAEPA), Brazil.

Acknowledgments

The authors thank Dr Aguinaldo Luiz Simões for providing the authentication of the cell line.

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Received in final form 1 April 2016

Accepted 16 May 2016

Accepted Preprint published online 16 May 2016