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EXPRESSION OF INSULIN-LIKE GROWTH FACTOR-II AND ITS RECEPTOR IN PEDIATRIC AND ADULT ADRENOCORTICAL TUMORS

Yoosef Hussain Alzaydey[1]*, Saif Hussien Alzaydey[2], Omar Abdulaziz Al-Nasser[3], Norah hamad Almogri[4], Essa Abdulrahman Al Mutairi[5], Satam Essa Alhamdan[6], Yousef Mushahhin almutairi[7], Waladin Faiz Mahrus[8], Dina Abdulwahab Abumelha[9]

[1]Assistant Pharmacist, KFMC, Riyadh, Sa.
[2]Assistant Pharmacist Dr. S KFMC, Riyadh, Sa.
[3,4]Pharmacist, KFMC, Riyadh, Sa.
[5,6]Staff Nurse, KFAFH, Riyadh, Sa.
[7]Technician Pharmacy, KFAFH, Riyadh, Sa.
[8]Pharmacist II, KFAFH, Riyadh, Sa.
[9]Pharmacist Tech, KFAFH, Riyadh, Sa.

***Corresponding Author: Yoosef Hussain Alzaydey**
Assistant Pharmacist, KFMC, Riyadh, Sa.

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ABSTRACT

Introduction: Adrenal cortical tumors are heterogeneous neoplasms whose pathogenesis is not fully understood. IGF-II overexpression has been consistently demonstrated in adult adrenal carcinomas. **Objectives:** The aim of the study was to analyze the expression of IGF-II and its receptor (IGF-IR) in pediatric and adult adrenal tumors and the effect of a selective IGF-IR kinase inhibitor (NVP-AEW541) on adrenal cortical tumor cells. patients: 57 adrenal tumors (37 adenomas and 20 carcinomas) from 23 children and 34 adults were examined. **Methods:** Gene expression was determined by real-time quantitative PCR. Cell proliferation and apoptosis were analyzed in NCI H295 cells and in a new cell line derived from a pediatric adrenal adenoma. **Results:** IGF-II transcripts were overexpressed in both adrenal cortical carcinoma and infantile adenoma. Otherwise, IGF-II was predominantly overexpressed in adult adrenal carcinomas (270.5 ±, 130.2 vs.16.1±13.3; P = 0.0001). IGF-IR expression was significantly higher in pediatric adrenocortical carcinomas than in adenomas (grades 9-12).1±3.1 versus 2.6±0.3; P=0.0001), while its expression was similar in adrenocortical carcinomas and in adult adenomas. IGF-IR expression was a predictor of adrenal tumor metastasis in children in a univariate analysis (relative risk 1.84; 95% confidence interval 1.28-2.66; p=0, 01). Furthermore, NVP-AEW541 dose- and time-dependently blocked cell proliferation in both cell lines by significantly increasing apoptosis. **Conclusion:** IGF-IR overexpression was a biomarker for childhood adrenal carcinoma. Furthermore, a selective IGF-IR kinase inhibitor demonstrated antitumor effects in adult and pediatric adrenal- cortical cancer cell lines, suggesting that IGF-IR inhibitors represent a promising therapy for human adrenal-cortical carcinoma.

INTRODUCTION

Adrenocortical carcinomas account for only 0.05– 0.2% of all cancers, with an estimated incidence of 0.5–2 per million per year in adults.[1– 4] Differently from adults, pediatric adrenocortical tumors with apparently poor prognosis based on the histopathological features often have a better clin- ical outcome.[8,9] To date, there are limited data to define histological or molecular markers that can reliably distinguish benign from malignant adrenocortical tumors, mainly in pedi- atric patients.[10] The molecular pathogenesis of adrenocortical tumors is still poorly understood. The IGF system has an essential role in nor- mal adrenocortical cell growth and development.[11,12,13–15] demon- strated that structural rearrangement of the 11p15 locus, typi- cally uniparental paternal isodisomy, and IGF-II overexpression were found in the great majority of adult sporadic adrenocortical carcinomas. However, the role of IGF-IR in adult and pediatric adrenocortical tumorigenesis re- mains to be determined. Cytotoxic chemotherapy has been extensively used for met- astatic adrenocortical carcinoma, although response rates are generally por.[3,18,19] is clear that current treatment protocols are not effective and that new therapies are strongly needed.[20] Two microarray studies identified that up-regulation of IGF-II expression was the dom- inant change in malignant adrenocortical tumors.[21,22] Con- sequently, IGF-IR inhibition has been proposed as the most appropriated target for adrenocortical carcinoma treatment.[16,20,23] In this study, we investigated IGF-II and IGF-IR expression in pediatric and adult adrenocortical tumors. In addition, the effects of the

NVP-AEW541 on proliferation and apoptosis were analyzed in NCI H295 cells and a new cell line that was estab- lished from a pediatric adrenocortical adenoma of our cohort.

PATIENTS AND METHODS

The study was approved by the ethics committee of Hospital das Clinicas, Sao Paulo, Brazil, and written informed consent was obtained from all patients and/or parents. The clinical and histopathological features of children and adults with adrenocortical tumors are summarized in Supplementary Tables 1 and 2, respectively, published as Supplementary Data on the Journal's online website Journal of the Endocrine Society at http://jcem.endojournals.org. Sporadic adrenocortical tumor samples were obtained from 23 children (16 girls and 7 boys; 0.9 to 15 years old) and 34 adult patients (29 women and 5 men; 18 to 66 years old). The mean follow-up time was 57.8 ± 6.2 months. In the pediatric group, we observed that 7 out of 13 tumors with a Weiss score of at least 4 had a benign progression, confirming that this particular criterion is not reliable for classifying pediatric tumors.[9] Thus, the diagnosis of malignancy in this group was established in six of the 23 pediatric adrenocortical tumors according to advanced tumor stage (III or IV) and/or poor clinical course. Mature adrenocortical tumors were classified according to the Weiss criteria: 20 cortical adenomas (Weiss score ≤3) and 14 carcinomas (Weiss score ≥4).[26] The p53 tumor suppressor gene was previously studied in 22 children and 26 adults, and the known Arg337His mutation was identified in 77 and 12% of them, respectively.[7,27]

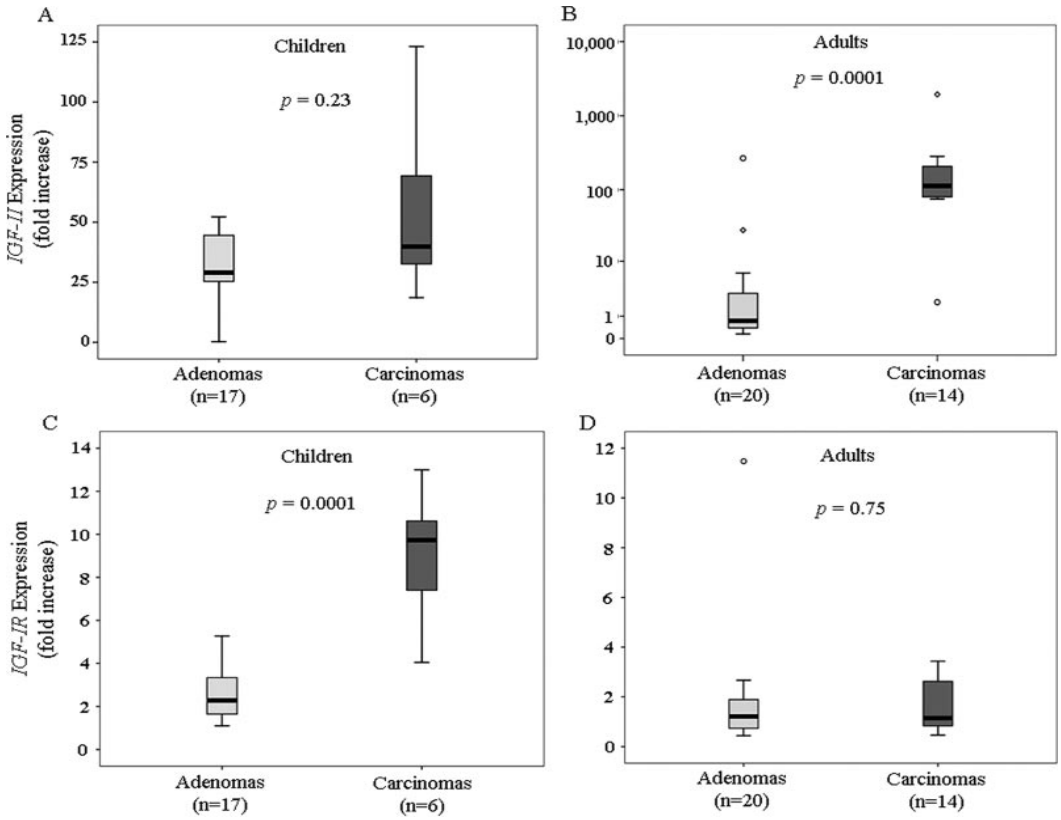

Fig. 1: Expression of IGF-II (A and B) and IGF-IR (C and D) in 57 sporadic adrenocortical tumors (23 children and 34 adults) using quantitative real-time PCR. Y axis shows fold increase in IGF-II and IGF-IR expression of tumor samples relative to the mean IGF-II and IGF-IR expression levels of a pool of normal adrenals. The horizontal line within the box plot represents the median value, the box plot limits refer to 25th to 75th percentiles, and the box plot bars includes the 10th to 90th percentiles for mRNA levels.

Quantitative real-time PCR

After surgical resection, tumor fragments were immediately frozen in liquid nitrogen and stored at -80 C until total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was generated from 1 µg total RNA using the High Throughput Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in an ABI Prism 7700 sequence detector using a TaqMan gene expression assay for gene quantification according to the manufacturer's instructions (Applied Biosystems). The test identifiers are: IGF-II, Hs01005963_m1; IGF-IR, Hs00181385_m1; -actin, 43263; 3-β-hydroxysteroid dehydrogenase type II (HSD3B2) Hs00605123_m1; 11-β-hydroxylase (CYP11B1) Hs01596404_m1; 21-hydroxylase (CYP21A2) Hs00416901_g1. Cyclic threshold (CT) values in the linear amplification range were selected for each sample in triplicate and normalized to β-actin expression levels.Relative expression levels were analyzed by 2-ΔΔCT method, where ΔΔCT was the difference between the selected ΔCT value of a particular sample and the ΔCT of the group of 61 normal adrenal

glands from autopsy (CLONTECH, Palo Alto, CA) (28). The mean expression of target genes in the normal adrenal region was assigned an expression value of 1, and increased expression levels were determined for each tumor sample and adrenocortical tumor cell lines.

Adrenocortical cell lines

The NCI H295 cell line, previously established from an invasive pri- mary adrenocortical carcinoma, was kindly provided by Dr. Walter L.

Steroid hormone analysis

Steroid secretion was measured in 5 d clarified supernatant medium of pediatric tumor cell culture by commercial kits: cortisol and testos- terone, fluorometric assay (AutoDELFIA; Wallac, Oy, Finland); andro- stenedione, chemiluminescent enzyme immunoassay (Immulite 2000; Siemens, Siemens Medical Solutions Diagnostics, Los Angeles, CA); 17-OH progesterone, RIA (Diagnostic Systems Laboratories, Webster, TX).

Immunocytochemistry analysis

Approximately 1–2 × 104 cells were seeded on cover plates in DMEM containing 10µS and fixed with 4% formaldehyde for 20 min. Immunohistochemical staining was performed using antibodies against vimentin (mouse monoclonal, 1:100; Novocastra, Newcastle upon Tyne, UK) and Melan A/mart 1 (clone) monoclonal mouse resistant A103; Chemicon, Temecula, CA). Immunocomplexes were detected by immunoperoxidase staining using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine as previously described (30). Cultured cells from normal human skin fibroblasts and the human melanoma cell line LB373-MEL were used as positive controls for vimentin and melan-A, respectively. Cell cultures incubated in non-immune primary antibodies were negative

A selective IGF-IR kinase inhibitor (NVP-AEW541)

NVP-AEW541, a pyrrolo[2,3-*d*]pyrimidine derivate highly selec- tive against IGF-IR, was kindly provided by Novartis Pharma (Basel, Switzerland) (24). Stock solution of this drug was prepared in di- methylsulfoxide and stored at —20 C.

Cell proliferation and caspase-3/7 activity assays

Cortical tumor cell lines were plated in 96-well plates at a density of 20,000 cells/well. cortical tumor cell lines were plated in 96-well plates at a density of 20,000 cells/well. OD was measured at 450 nm in an ELISA reader.The programmed death assay was based on caspase-3/7 activity after treatment with, µg/ml deoxyribonuclease I (Life Technologies, Inc., Paisley, UK), mechanical degradation for 30 min, and movement. Two media were used: DMEM and MEM serum medium-modified reduction (Opti-MEM I) supplemented with 10 and 2 µtal bovine serum (FBS) and 1% penicillin/streptomycin, respectively. Two media were used: DMEM and MEM serum medium-modified reduction (Opti-MEM I) supplemented with 10 and 2 µtal bovine serum (FBS) and 1% penicillin/streptomycin, respectively. The initial growth of, at a slow rate, occurred in both media and regardless of the medium used, the cells were adherent and spindle-shaped.Cells were cultured in DMEM supplemented with 10µS for growth and steroid secretion studies. The initial growth of, at a slow rate, occurred in both media and regardless of the medium used, the cells were adherent and spindle-shaped.Cells were cultured in DMEM supplemented with 10µS for growth and steroid secretion studies. The morphological features of pediatric adrenocortical adenoma cells were examined under phase-contrast microscopy and light microscopy after staining with hematoxylin and eosin.

The morphological features of pediatric adrenocortical adenoma cells were examined under phase-contrast microscopy and light microscopy after staining with hematoxylin and eosin. Adrenal cortical tumor cell lines were maintained at 37°C in medium fully humidified at 95% air and 5% CO and cultured in DMEM medium containing 10S and 1% penicillin/streptomycin. Adrenal cortical tumor cell lines were maintained at 37°C in medium fully humidified at 95% air and 5% CO and cultured in DMEM medium containing 10S and 1% penicillin/streptomycin. All in vitro experiments and steroid analyzes were performed in the fifth passage of the pediatric adrenocortical adenoma cell line. All in vitro experiments and steroid analyzes were performed in the fifth passage of the pediatric adrenocortical adenoma cell line. Repeated measurements of absorbance and luminescence were compared using ANOVA followed by Bonferroni's post hoc test. Repeated measurements of absorbance and luminescence were compared using ANOVA followed by Bonferroni's post hoc test. The significance level for the adjusted Bonferroni tests was set at 0.0024. The significance level for the adjusted Bonferroni tests was set at 0.0024.

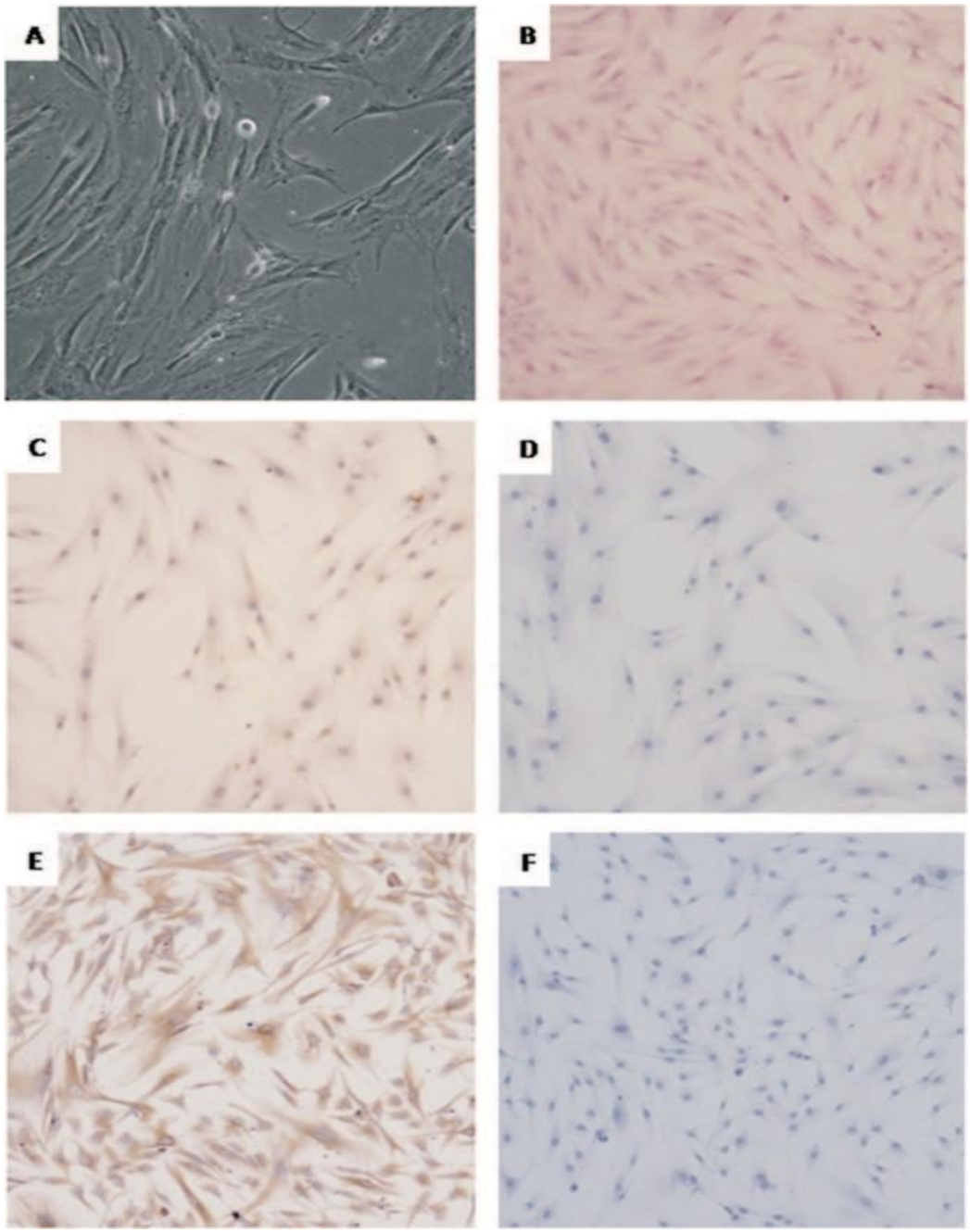

Fig. 2: The pediatric adrenocortical adenoma cells had a fibroblastoid and spindle-shaped appearance at phase contrast microscopy (A, ×10 magnification) and hematoxylin and eosin staining (B). A positive immunoreactivity for melan-A, a melanocytic differentiation marker, was detected by immunocyto- chemistry in 100% of the pediatric tumor cells (C, melan-A immunoreactivity; D, negative control cells). A strong cytoplasmic expression of vimentin, an intermediate filament of mesenchymal cells, was also evidenced in 100% of these adrenocortical cells (E, vimentin immunoreactivity; F, negative control cells).

RESULTS

IGF-II and IGF-IR expression

IGF-II transcripts overexpressed in both pediatric adrenocortical carcinoma and adenoma (50.8 ± 18.5 vs 31.2 ± 3.7, respectively; P=0.23) (Figure 1A). On the other hand, IGF-II was mainly overexpressed in adult adrenocortical carcinomas, compared with adenomas (270.5 ± 130.2 vs. 16.1 ± 13.3; P = 0.0001 (Fig.1B) according to previous studies (14, 15, 22). IGF-IR mRNA levels were significantly higher in pediatric adrenocortical carcinomas compared with adenomas (9.1 ± 3.1 vs 2.1).6±0.3; P = 0.0001), while similar levels of IGF-IR expression were detected in adrenocortical carcinomas and adult adenomas (1.6 ± 0.3 vs. ± 0.3).1.8 ± 0.5 respectively; P = 0.75) (Figures 1, C and D).Metastasis was noted in six of 23 children and seven of 34 adults with adrenocortical tumors. In pediatric adrenocortical tumors, tumor weight [RR 16.6, 95% confidence interval (CI) 1.54, 178.9; p=0.02], histopathological characteristics of malignancy (Weiss

score) (HR 2.11; 95% CI 1.2–3.72; P=0.009) and IGF-IR mRNA level (HR 1.84; 95% CI 1.28 -2.66; P = 0.01) was associated with a higher risk of metastatic disease in the univariate analysis (Table 1). In adult adrenal cortical tumors, male sex (HR 9.96; 95% CI 1.93–51.29; P=0.006), tumor weight (HR 6.39; 95% CI 1.44 -28.36; P = 0.01) and Weiss score (HR 1.74; 95% CI 1.24 -2.44; P = 0.015) was associated with a higher risk of metastatic disease in univariate analysis. IGF-II, ment with NVP-AEW541 (0.3–30 μM). After 3 to 9 h of treatment, cells were incubated with Caspase-Glo 3/7 Assay (Promega) for 1 h and luminescence signal was measured with a luminometer. All cell proliferation and apoptosis experiments were performed in triplicate.

Statistical analysis

All statistical analyses were performed with the SPSS software (SPSS 13.0; SPSS, Inc., Chicago, IL). Continuous data are expressed as mean ± SEM. Differences in expression levels between adenomas and carcinomas were analyzed by means of the two-tailed Mann-Whitney *U* test. Pre- dictive factors of metastases were identified by means of Cox proportional hazards regression models, which was used to estimate hazard ratios (HR) and their 95% confidence intervals in univariate analysis.

Characterization of the new pediatric adrenocortical tumor cell line

To dissect the cellular consequences of IGF-IR inhibition in pediatric and adult adrenocortical tumors, two adrenocortical cell lines were studied. The NCI H295 cell line was previously obtained from an invasive primary adrenocortical carcinoma in a 48-yr-old woman (29). Here we obtained a new pediatric adrenocortical tumor cell culture from a functioning ad- renocortical adenoma. The pediatric adrenocortical adenoma cell line had a fibro- blastoid and spindle-shaped appearance at phase-contrast microscopy and hematoxylin and eosin staining, respectively (Fig. 2, A and B). Pediatric adrenocortical adenoma cell cul- ture showed cytoplasmic immunoreactivity for melan-A in 100% of the culture cells (Fig. 2, C and D). The melan-A is a melanocytic differentiation marker, which has the useful property of staining steroid hormone-producing tumors, such as adrenocortical adenomas and carcinomas (31). A strong cytoplasmic expression of vimentin, the major intermediate filament protein of mesenchymal cells, was also detected in 100% of these adrenocortical cells (Fig. 2E and F). This homogeneity pattern of this cell culture suggests one cellular type isolated from the tumor fragments.

NVP-AEW541 effects on adrenocortical tumor cell lines

To achieve this goal, we evaluated the effects of NVP-AEW541 on blocking IGF-II stimulated proliferation of human NCI H295 and pediatric adrenocortical adenoma cells.

IGF-II significantly increased proliferation of NCI H295 and pediatric adrenocortical adenoma control cells after 72 and 48 h, respectively (P = 0.0001). The NVP-AEW541 treatment had a significant effect on proliferation reduction of NCI H295 cells at increasing concentrations of 10 μM (70 ± 10%) and 30 μM The treatment with this IGF-IR inhibitor significantly decreased NCI H295 cell proliferation at 0.3 μM (70.3 ± 2.7%), 1.0 μM (51.4 ± 5.4%), 3.0 μM (35.1 ± 2.7%), NVP- AEW541 treatment promoted a near-total reduction in NCI H295 cell proliferation at 10 and 30 μM after 96 h (Fig. 3A). NVP-AEW541 also promoted a significant decrease of pediatric adrenocortical adenoma cell proliferation at 0.3 μM (81.2 ± 3%), 1 μM (72.3 ± 6%), 3 μM (63.4 ± 6%), 10 μM (2 ± 0.3%),

This IGF-IR inhibitor led to a progressive reduction on proliferation of pediatric adrenocortical adenoma cells at 0.3 μM (78.2 ± 5.6%), 1.0 μM (61.3 ± 1.6%), (1.6 ± 0.2%), compared with untreated cells (100 ± 1.6%) at 96 h (P = 0.0001) (Fig. 3B). NVP-AEW541 treatment of both adrenocortical tumor cell lines without exogenous IGF-II stim- ulation also promoted a significant reduction in cell proliferation (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http:// jcem.endojournals.org). 0.06 μM for NCI H295 and for pediatric adrenocortical ade- noma cells after 96 h of treatment, respectively. The NCI H295 cells were more sensitive to NVP-AEW541, showing IC50 value at a submicromolar concentration. NCI H295 cells treated with NVP- AEW541 showed a significant increase in caspase-3/7 activity at RLU), compared with untreated cells (2019 ± 329 RLU) at 3 h (P = 0.0001) (Fig. 4A).

The IGF-IR inhibition in pediatric adrenocortical adenoma cells significantly increased caspase-3/7 activity at 3.0 μM (3220.4 ± 56.2 RLU), 10 μM (4056 ± 277.9 RLU), and 30 μM (6324.6 ± 198.3 RLU), compared with un- treated cells (1732.9 ± 61.4 RLU) at9h (P = 0.0001) (Fig. 4B).

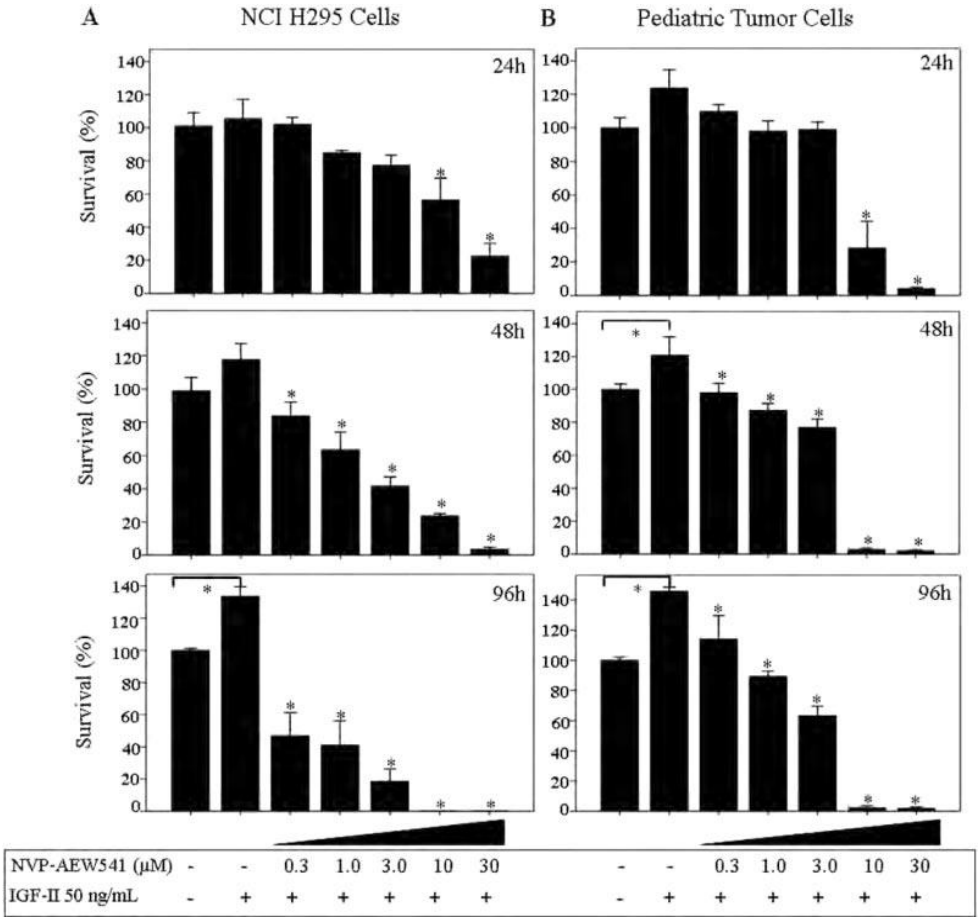

Fig. 3: The NVP-AEW541 treatment had a significant effect on proliferation reduction of NCI H295 cells at increasing concentrations of 0.3–30 µM after treatment for 24 –96 h (A). This IGF-IR inhibitor also led to a significant decrease of the human pediatric adrenocortical adenoma cell proliferation after treatment for 24 –96 h (B). Tumor cells treated with NVP-AEW541 were compared with untreated cells stimulated by IGF-II. Data are expressed as mean ± SEM of triplicates.*, P = 0.0001.

DISCUSSION

Binding of ligands to the IGF-IR or its own overexpression initiates a cascade of events, leading to stimulation of proliferation, angiogenesis, apoptosis, and inhibition of metastases (32). A strong overexpression of IGF-II is a dominant finding in adult adreno- cortical cancer, occurring in approximately 83% of malignant adrenocortical tumors (14). Nonetheless, very few studies about IGF-II expression in pediatric adrenocortical tumors were re- ported (35, 36). We demonstrated that IGF-II expression was deregulated in a similar manner in both pediatric adrenocortical adenomas and carcinomas. IGF-IR overexpression was previously demonstrated in adult adrenocortical carcinomas but not adrenocortical hyperplasias and adenomas (37, 38). In this study, we identified that IGF-IR expression was similar in benign and malignant adult adreno- cortical tumors. Otherwise, a strong increase in IGF-IR expression was identified only in pediatric adrenocortical carcinomas.

In our cohort, IGF-IR expression was a predictor of metastases in children with adrenocortical tumors. The mechanisms respon- sible for enhanced IGF-IR expression in pediatric adrenocortical tumors are still unclear.

Changes in IGF system expression in cancerous cells may occur as a result of loss or altered expression of tumor suppressor genes.[17] In normal cells, expression of wild-type P53 was shown to inhibit IGF-IR expression, whereas mutant P53 up-regulates IGF-IR expression in different tumors.[39] Nevertheless, IGF-IR expression levels were not associated with the presence of this mutation (data not shown).

Therefore, other molecular events, like IGF-IR gene amplification, may be involved in IGF-IR overexpression in pediatric adrenocortical tumors.

Analysis of tumor-derived cell lines provides an effective system for establishing the link between specific tumor molecular aspects and the response to molecular target drugs.[40] We established a new transitory cell culture derived from a human pediatric adrenocortical adenoma, thus permitting the study of a specific signaling pathway that may interfere with adrenocortical tumor growth in children.

We demonstrated that NVP-AEW541, a se- lective IGF-IR kinase inhibitor, was able to block cell prolifer- ation in a dose- and time-dependent manner in two distinct human adrenocortical tumor cell lines. Furthermore, NVP-AEW541 also inhibits cell cycle progression, inducing specific G1 arrest.[25,42]

The level of sensitivity of the pediatric adrenocortical tumor cells was similar to that of hepatocellular carcinoma and gastrointestinal tumor cells.[42,43]

In conclusion, IGF-IR overexpression was a biomarker of pediatric adrenocortical carcinomas. In addition, we demon- strated that a selective IGF-IR kinase inhibitor had antitumor effects in adult and pediatric adrenocortical tumor cell lines, suggesting that IGF-IR inhibitors represent a promising therapy for human adrenocortical carcinoma.

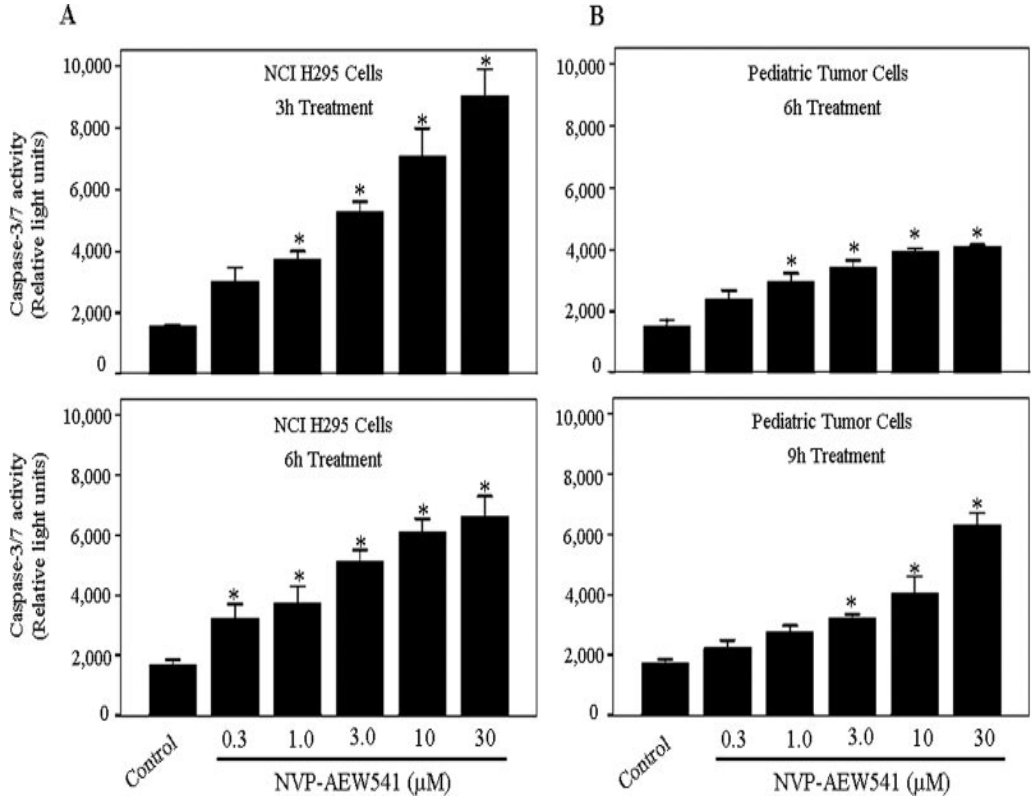

Fig. 4: NVP-AEW541 induced apoptosis by a significant increase of caspase-3/7 activity in NCI H295 cells at concentrations of 0.3–30 μM after 3 and 6 h (A). NVP- AEW541 also increased caspase-3/7 activity in pediatric adrenocortical adenoma cells after treatment for 6 and 9 h (B). Tumor cells treated with NVP-AEW541 were compared with untreated cells. Data are expressed as mean ± SEM of triplicates.*, P = 0.0001.

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