Insulin-Like Growth Factor and *SLC12A7* Dysregulation: A Novel Signaling Hallmark of Non-Functional Adrenocortical Carcinoma

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BACKGROUND: Insulin-like growth factor (IGF) dysregulation and gene copy number variations (CNV) are hallmarks of adrenocortical carcinoma (ACC). The contribution of IGF CNVs in adrenal carcinogenesis has not been studied previously. In addition, studies demonstrating an association between *SLC12A7* gene amplifications and enhanced metastatic behavior in ACC, as well as reported IGF-SLC12A7 signaling interactions in other cancers, suggest a potential IGF-SLC12A7 signaling circuitry in ACC. Here we investigate the potential complicity of IGF-SLC12A7 signaling in ACC.

STUDY DESIGN: Insulin-like growth factor CNVs were determined by whole-exome sequencing analysis in an exploratory cohort of ACC. Quantitative polymerase chain reaction methods determined *IGF1* and *IGF2* expression levels and were evaluated for correlation with *SLC12A7* expression and tumor characteristics. Insulin-like growth factor CNVs and expression patterns were compared with The Cancer Genome Atlas. In vitro studies determined the relationship of IGF and *SLC12A7* co-expression in 2 ACC cell lines, SW-13 and NCI-H295R. Immunohistochemistry assessed IGF1 receptor (IGF1R) activation.

RESULTS: The *IGF1* gene was amplified in 9 of 19 ACC samples, similar to findings in The Cancer Genome Atlas database. The *IGF1* overexpression was observed in 5 samples and was associated with *SLC12A7* overexpression and non-functional, early-stage tumors ($p < 0.05$). In contrast, *IGF2* overexpression was associated with larger tumors ($p < 0.05$). In vitro IGF treatment of ACC cell lines did not stimulate *SLC12A7* expression, and endogenous overexpression and silencing of SLC12A7 significantly altered *IGF1* and *IGF1R* expression without impacting other IGFs. The IGF1R activation was associated with *IGF1* overexpression in ACC tumor samples.

CONCLUSIONS: These findings indicate that *IGF1* overexpression, caused in part by gene amplifications, is correlated with *SLC12A7* overexpression in non-functional, early-stage ACCs, suggesting a potentially targeted IGF1-SLC12A7 therapeutic opportunity for these tumors. (J Am Coll Surg 2019;229:305–315. © 2019 Published by Elsevier Inc. on behalf of the American College of Surgeons.)

Adrenocortical carcinoma (ACC) is a rare cancer of the adrenal cortex, with a reported incidence of 0.7 to 2 cases per million people per year.[1] Despite recent advancements in our understanding of the molecular causes of adrenocortical tumorigenesis, little improvement has been made in overall treatment outcomes, especially in those patients with advanced and/or metastatic diseases. The overall prognosis for ACC is poor, and a recent

Disclosure Information: Nothing to disclose.

Support: This study was supported by an Ohse Research Grant.

Presented at the 99th Annual Meeting of the New England Surgical Society, Portland, ME, September 2018.

Received March 13, 2019; Accepted April 11, 2019.

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https://doi.org/10.1016/j.jamcollsurg.2019.04.018
ISSN 1072-7515/19

Abbreviations and Acronyms

ACC = adrenocortical carcinoma
CNV = copy number variations
HRP = horseradish peroxidase
IGF = insulin-like growth factor
IGF1R = insulin-like growth factor 1 receptor
mRNA = messenger RNA
SLC12A7 = solute carrier family 12 member 7
TCGA = The Cancer Genome Atlas
WES = whole-exome sequencing

analysis of the National Cancer Data Base demonstrated a 5-year survival for stage IV tumors to be <50%.[2] In addition, recurrence rates can be very high after curative intent resections, indicating the need for improved adjuvant therapies after R0 resection.[3]

The 3 most common molecular alterations observed in ACC include dysregulation of WNT, TP53, and insulin-like growth factor (IGF) signaling pathways.[4] A gain-of-function mutation of the β-catenin gene (*CTNNB1*), a proto-oncogene, promotes its nuclear persistence by inhibiting its programmed cytoplasmic degradation by a destruction complex. This in turn enhances constitutive target gene transcription and tumor formation.[5] Germline *TP53* mutations are frequently found in childhood cases of ACC,[6,7] and somatic *TP53* mutations occur commonly in aggressive adult tumors and are believed to represent a late event in adrenal carcinogenesis.[8] However, the most frequent molecular event observed in ACC and an area of intense interest for developing targeted therapies, is the overexpression and activation of the IGF signaling system.

In normal adrenal tissue, IGF2 expression is highest during embryogenesis, and IGF1 expression occurs primarily after birth. Both IGFs bind the IGF1 receptor (IGF1R) and promote adrenal growth and cortisol production.[9-12] In adrenal malignancy, IGF2 is the predominant IGF overexpressed, driven in part by alterations in methylation patterns, and is associated with more-aggressive tumors. This is best exemplified by the development of pediatric ACCs in Beckwith-Wiedemann syndrome, a genetically heterogeneous condition due to epigenetic alterations on chromosome 11p15.5, which causes constitutive IGF2 upregulation in subsets of patients. A few studies also report heightened expression of IGF1 in ACC, implicating a global role for the IGF system in adrenal tumorigenesis.[13,14] Interestingly, neither overexpression of *IGF1* or *IGF2* alone has been shown to cause malignant transformation in the adrenal gland.[9] Apart from changes in promoter methylation of the *IGF2* gene in a subset of ACCs, the cause(s) of aberrant IGF signaling observed frequently in ACC is poorly understood. Gene copy number alterations, a common genome-wide phenomenon in ACC,[15] have not been specifically analyzed at the IGF loci and are studied here.

Other than its known role in promoting cellular growth, the underlying mechanisms by which aberrant IGF signaling potentially modulates a more aggressive phenotype in ACC has not been fully clarified. Recent studies identified potassium chloride transporter, *solute carrier family 12 member 7* (*SLC12A7*) overexpression as one of the aberrant processes that promotes the aggressive behavior of ACCs via alterations in membrane architecture and invasive properties.[16,17] Interestingly, co-expression of IGF1 and SLC12A7 has been observed in several gynecologic tumor types, especially in metastatic lesions. In addition, tumor cell stimulation with IGF1 was associated with SLC12A7 membrane localization and increased tumor cell invasion kinetics.[18] We hypothesize that a similar invasion-promoting role for SLC12A7 in ACC is potentially promoted, in part, by deregulated IGF signaling and is investigated here.

METHODS

Study cohort

After approval by the Yale University and Karolinska Institute IRBs, 33 cases (8 Yale, 25 Karolinska) of histologically confirmed ACCs were selected for biochemical and clinical analysis. Clinical characteristics of the patients are shown in Table 1. All fresh-frozen adrenal tissues samples were maintained in a prospectively maintained endocrine tumor repository and experienced endocrine pathologists reviewed tissue sections for confirmation of the diagnosis before investigation.

Tumor gene copy analysis

Genomic DNA from ACC samples was recently subjected to whole-exome sequencing (WES) in which chromosomal, arm-level copy number variations (CNV) were reported by Juhlin and colleagues.[19] A separate reanalysis was performed with an alternative algorithm to identify single-gene CNVs for the IGF loci on chromosome 11 and 12 in 19 samples by assessing coverage depth analysis of WES reads between tumor and adjacent normal adrenal DNA using the genomic identification of significant targets in cancer (ie GISTIC [Genomic Identification of Significant Targets in Cancer], version 2.0), algorithm output for gene-level data. A larger confirmatory cohort from The Cancer Genome Atlas (TCGA) database was analyzed (Xena Browser; University of California Santa Cruz) to determine gene copy alterations of *IGF1* and *IGF2* as well.[20]

Tumor gene expression analysis

RNA was isolated from fresh-frozen samples using the AllPrep DNA/RNA/Protein Kit (Qiagen). Quantity and

Table 1. Association of Insulin-Like Growth Factor Expression Levels and Patient Characteristics

Characteristic	Data (n = 33)	*IGF1* expression, p value	*IGF2* expression, p value
Sex, n	—	0.43	0.20
Male	12		
Female	21		
Age, y, mean ± SD	57.6 ± 13.8	0.56	0.28
Tumor size			
Diameter, cm, mean ± SD	13.0 ± 4.4	0.40	0.08
Weight, g, mean ± SD	755 ± 667.1	0.95	0.01*
ENSAT stage, n	—	0.01*	0.71
I to II	17		
III to IV	16		
Hormone status, n	—	0.01*	0.78
Aldosterone	1		
Cortisol	8		
Androgen	3		
Multi-secreting†	5		
Non-functional	12		
Unknown	4		
Outcome, n	—	0.26	0.74
Alive, no recurrence	10		
Alive, recurrent	3		
Death from disease	16		
Death from other cause	4		

*Statistically significant.
†Tumors secreting 2 or more of the following hormones: aldosterone, cortisol, testosterone, or dehydroepiandrosterone.
ENSAT, European Network for the Study of Adrenal Tumors.

quality of isolated RNA was assessed by spectrophotometry (NanoDrop Technologies) and 200 ng RNA was used for complementary DNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative polymerase chain reaction was performed on a CFX96 Real-Time System thermal cycler (Bio-Rad) using TaqMan PCR master mix with primers and probes (Applied Biosystems) specific to *IGF1* (*Hs01547656_m1*), *IGF2* (*Hs04188276_m1*), *SLC12A7* (*Hs00986431_m1*), and the housekeeping gene *large ribosomal protein 0* (*RPLP0; Hs00420895_gH*). Relative expression levels were calculated using the Livak method.[21] The normal reference tissue analyzed in this study for comparison included 10 samples of histologically normal adrenal tissue surgically removed, along with adjacent adrenal adenoma samples. A larger confirmatory cohort analyzing the TCGA database RNA sequence reads (Xena Browser) determined gene expression levels of *IGF1* and *IGF2*. Assays were performed in triplicate.

Cell culture

The authenticated ACC cell lines SW-13 and NCI-H295R were purchased from the American Type Cell Collection and maintained per American Type Cell Collection protocol, as described previously.[17,22] Briefly, SW-13 cells were grown under sterile conditions in Dulbecco's modified Eagle's medium supplemented with 10% certified fetal bovine serum and 1% of 100× penicillin/streptomycin (ThermoFisher Scientific) in a standard humidified incubator at 37.0°C and 5% CO_2. NCI-H295R cells were grown under sterile conditions in Dulbecco's modified Eagle's medium/F12 supplemented with 5% NuSerum, 0.1% insulin-transferrin-selenium, and 1% of 100× penicillin/streptomycin (ThermoFisher Scientific) in a standard humidified incubator at 37.0°C and 5% CO_2.

In vitro insulin-like growth factor stimulation and expression

Two hundred and fifty thousand cells of SW-13 were seeded per well into a 6-well plate in 3 mL growth medium. After overnight incubation to allow for cell adherence, cells were then treated with various concentrations of recombinant IGF1 or IGF2. After 6, 12, and 24 hours of incubation, messenger RNA (mRNA) was isolated using RNeasy Plus Mini Kit (Qiagen) and mRNA levels of *SLC12A7* were determined. Similarly, mRNA expression levels of *IGF1*, *IGF2*, and *IGF1R* were also measured in

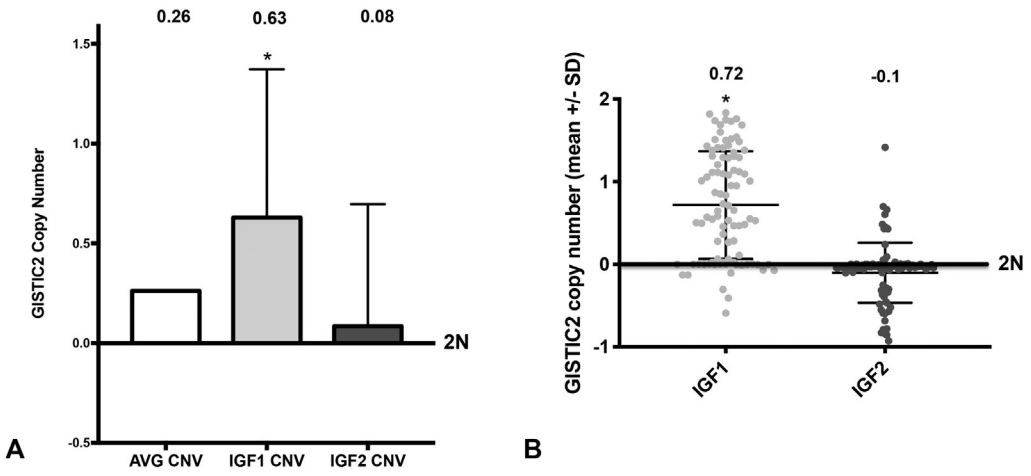

Figure 1. Insulin-like growth factor (IGF) gene copy analysis. (A) Nineteen samples that previously underwent whole-exome sequencing[19] were analyzed for gene copy alterations at the *IGF1* and *IGF2* loci. The *IGF1* gene was significantly amplified, ranking in the top 10% of all genes amplified using GISTIC2 (Genomic Identification of Significant Targets in Cancer, version 2) analysis (*$p < 0.05$; 2N, diploid). Mean value (base-2 logarithmic scale) is noted above each gene analyzed. (B) Analysis of The Cancer Genome Atlas adrenocortical carcinoma cohort database also demonstrated the *IGF1* gene to be significantly amplified, with minimal alterations observed in the *IGF2* gene (*$p < 0.05$; 2N, diploid). Horizontal bar, mean; error bars, SD. CNV, copy number variation.

SW-13 cells, which have low endogenous expression of *SLC12A7*, and in NCI-H295R cells, which have high endogenous expression of *SLC12A7*.[17] Assays were performed in triplicate.

SLC12A7 small interfering RNA knockdown and insulin-like growth factor expression

RNA interference gene silencing of NCI-H295R cells were carried out with 3 unique 27-mer small interfering

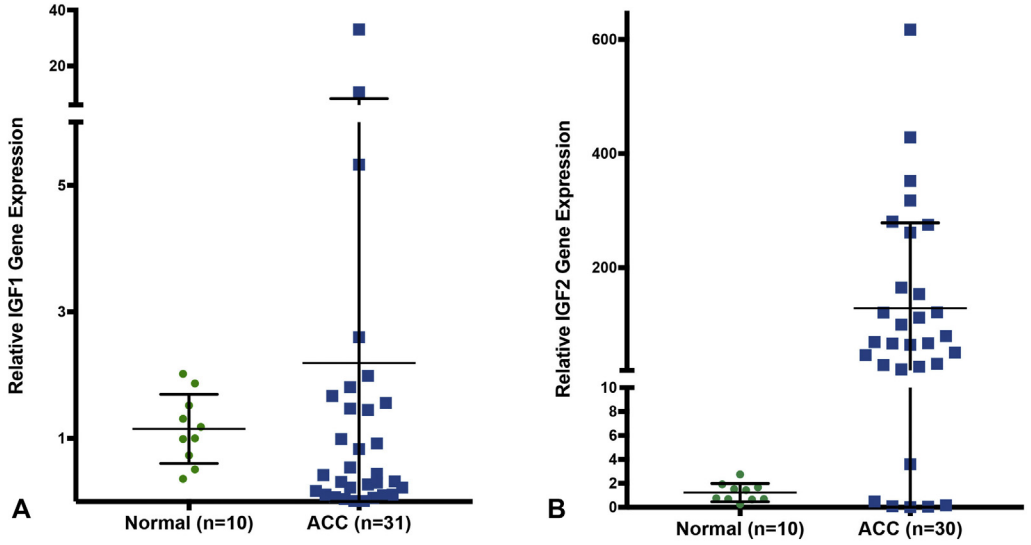

Figure 2. Insulin-like growth factor (IGF) gene expression analysis. (A) Relative messenger RNA expression levels of *IGF1* in adrenocortical carcinoma (ACC) samples (n = 31) were measured by real-time quantitative polymerase chain reaction (RT-qPCR) and compared with expression levels in normal adrenal tissues (n = 10). Five samples demonstrated a more than 2-fold increase in expression levels. (B) Relative messenger RNA expression levels of *IGF2* in ACC samples (n = 30) were measured by RT-qPCR and compared with expression levels in normal adrenal tissues (n = 10). Twenty-five of 30 samples demonstrated a more than 2-fold increase in expression levels. Horizontal bar, mean; error bars, SD.

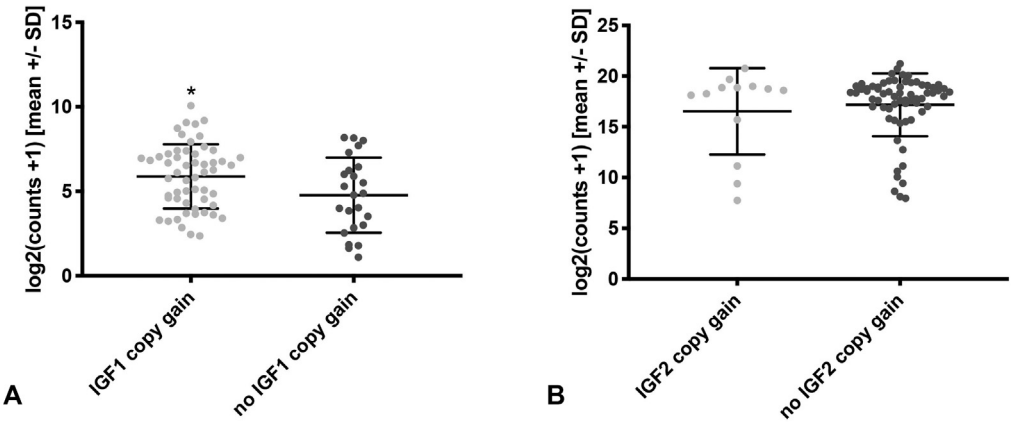

Figure 3. Insulin-like growth factor (IGF) gene expression analysis of The Cancer Genome Atlas (TCGA) adrenocortical carcinoma cohort. (A) *IGF1* expression levels were determined in the TCGA database and compared with TCGA *IGF1* gene copy alterations. Overall, higher gene expression was observed in samples with IGF1 gene amplifications (*$p < 0.05$). (B) *IGF2* expression levels were also determined and compared with *IGF2* gene copy alterations. No difference in expression levels was observed between IGF2-non-amplified and amplified genes. Horizontal bar, mean; error bars, SD.

RNA duplexes targeting *SLC12A7* (human) using the standard protocol, as described previously.[17] Universal scrambled small interfering RNA was used as a nonspecific control (all from Origene). Lipofectamine 3000-mediated transfection was carried out in Opti-MEM medium according to the manufacturer's recommendations (ThermoFisher Scientific) in 6-well plates with starting densities of 100,000 cells/well. Transfection medium was replaced with growth medium after 6 hours of transfection. Cells were lysed for RNA extraction and gene expression analysis at 24 hours post-transfection. The *IGF1* expression levels were determined in NCI-H295R cells undergoing small interfering RNA knock down of SLC12A7 expression, relative to the parental NCI-H295R cells that express high endogenous levels of SLC12A7. Confirmation of SLC12A7 overexpression or RNA interference knock down were confirmed by real-time quantitative polymerase chain reaction, as reported previously.[17] Assays were performed in duplicate.

Western blot detection of insulin-like growth factor 1 and insulin-like growth factor 1 receptor expression

Altered protein expression of IGF1 and IGF1R was confirmed via Western blot technique using goat anti-IGF1 and mouse monoclonal anti-IGF1R antibodies, followed by anti-goat-horseradish peroxidase (HRP) and anti-mouse-HRP antibodies (all from Invitrogen), mini-PROTEAN TGX gel (Bio-Rad), polyvinylidene fluoride blotting membrane (Bio-Rad), and enhanced chemiluminescence detection reagents (ThermoFisher Scientific) according to the manufacturer's protocols. Equivalent protein loading was ensured via Western blot detection of β-actin expression using anti-β-actin mouse monoclonal antibody (Santa Cruz Biotechnology) followed by anti-mouse-HRP secondary antibody (Invitrogen). Western blot analysis was performed in duplicate.

Immunohistochemistry

Representative sections of histologically confirmed ACCs and colon adenocarcinoma (positive control) from formalin-fixed, paraffin-embedded tissue samples were selected for study. Using immunohistochemistry methods per the manufacturer's protocol (Abcam), target epitopes were detected using anti-IGF1R (phosphoY1161) antibody at 1:200 dilution, followed by secondary antibody. 3,3′-diaminobenzidine tetrachydrochloride was used for antigen detection. Sections were counterstained with hematoxylin. Photomicrographs were taken at 400× magnification.

Statistical Analysis

A 2-tailed *t*-test or Mann-Whitney U test was used to assess difference in 2 groups with continuous distribution, for normal and non-normal variables, respectively. A 1-sample *t*-test was used to determine the significance of gene copy alterations. For variables with more than 2 dependent values, a 1-way ANOVA was used. Pearson correlation was used to compare matched continuous variables. Survival data were assessed by Kaplan-Meier methods and differences were compared by the Mantel-Cox test. Statistical analyses were performed using Prism 7 software (GraphPad). A p value < 0.05 was considered statistically significant.

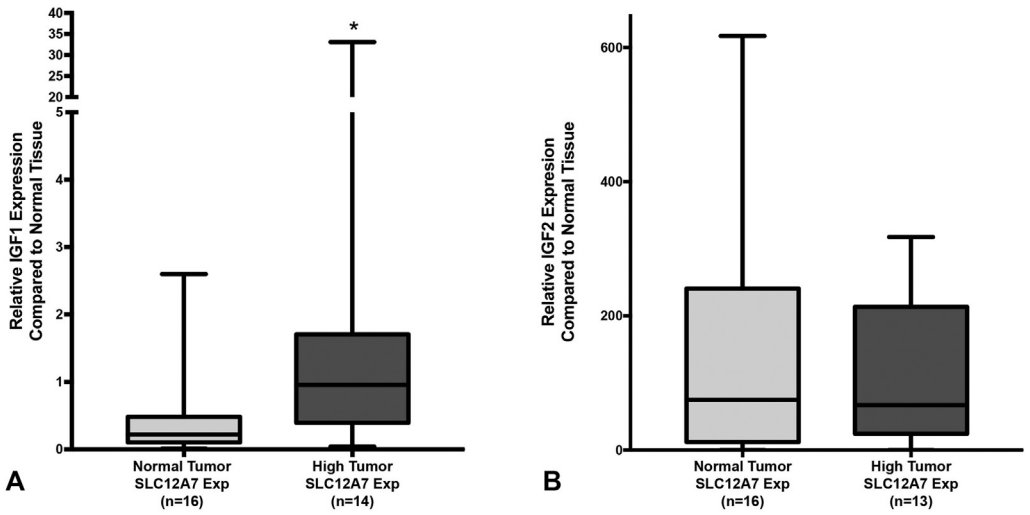

Figure 4. Insulin-like growth factor 1 (IGF1) and solute carrier family 12 member 7 (SLC12A7) Expression in adrenocortical carcinoma. (A) *IGF1* expression levels were assessed for possible correlation with *SLC12A7* expression, which was determined previously.[16] Normal adrenal tissue served as reference to calculate relative expression levels. Overexpression of *IGF1* and *SLC12A7* overexpression was correlated (*$p < 0.05$). (B) A similar association was not observed with *IGF2* and *SLC12A7* expression levels. Horizontal bar, mean; error bars, range.

RESULTS

Nineteen ACC and matched normal samples that were previously subjected to WES[19] were analyzed for gene copy alterations at the *IGF1* and *IGF2* loci. The *IGF1* gene was found significantly amplified, ranking in the top 10% of all the genes determined to be amplified, with 9 samples demonstrating a gain of at least 1 gene copy ($p < 0.05$, Fig. 1A). In contrast, the *IGF2* locus was not significantly amplified, with only 2 samples demonstrating gene copy gains. Analysis of the TCGA database also demonstrated the *IGF1* gene to be significantly amplified ($p < 0.05$, Fig. 1B), with mostly a copy-neutral status observed for the *IGF2* gene locus.

A total of 33 cases of ACC were analyzed for *IGF1* and *IGF2* gene expression using real-time quantitative polymerase chain reaction methods. RNA from 3 samples did not yield reliable amounts of complementary DNA for either *IGF1* or *IGF2* determination and were excluded from additional analysis wherever applicable. Ten samples demonstrated a relative increase in expression of *IGF1* compared with normal adrenal tissue, with 5 cases demonstrating than a more than 2-fold increase in expression levels. Overall relative expression levels were 2.2 times higher compared with normal adrenal tissue (Fig. 2A). This finding is in contrast to 2 previous reports that demonstrated unaltered *IGF1* expression in ACC tumors.[23,24] The *IGF2* expression was in accordance with previous findings and showed an average overall relative higher expression more than 100-fold. Only 5 samples in this cohort demonstrated decreased *IGF2* expression (Fig. 2B). Analysis of TCGA RNA sequence data showed that the majority of *IGF1* amplifications occurred in samples with *IGF1* mRNA overexpression ($p < 0.05$, Fig. 3A), and similar results were not shown for *IGF2* (Fig. 3B).

IGF expression patterns were assessed for correlation with tumor characteristics, including sex, age, tumor size, stage, hormonal status, and survival. Increased *IGF1* expression was found to be associated with non-functional tumors ($p < 0.05$, Table 1), with a nearly 8-fold increase in relative expression compared with hormonally active tumors. The *IGF1* expression was also 7 times higher in early-stage (European Network for the Study of Adrenal Tumors stage I to II) tumors ($p < 0.05$, Table 1). In contrast, *IGF2* expression was not associated with either hormone status or stage, but did correlate with tumor weight ($p < 0.05$, Table 1) and size ($p = 0.08$, Table 1). Increased *IGF1* expression was also found to be associated with *SLC12A7* overexpression ($p < 0.05$, Fig. 4A), but no similar correlation was observed with IGF2 (Fig. 4B). It should be noted that previous studies have analyzed the prognosis of IGF expression in ACC, but those findings were contradictory, most likely limited by their overall small cohort sizes.[25,26]

Previous studies have shown a role for IGF in promoting SLC12A7-mediated invasive activity in ovarian cancer cells, potentially through IGF1-induced SLC12A7 trafficking.[27]

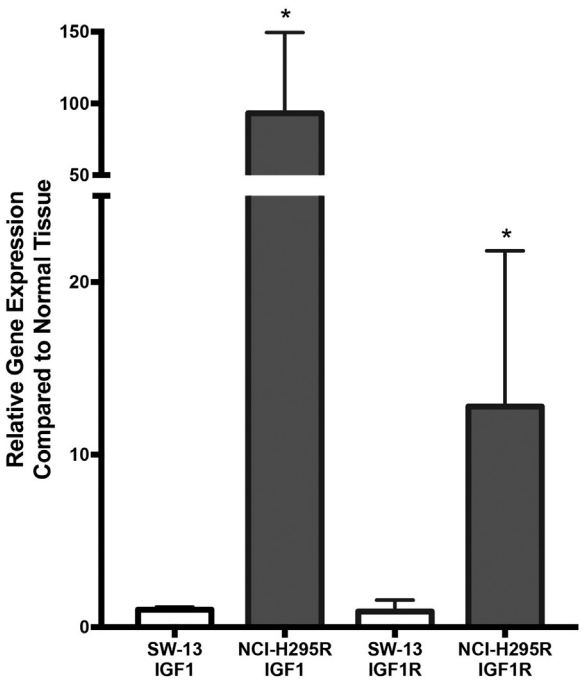

Figure 5. Insulin-like growth factor 1 (IGF1) and IGF1 receptor and solute carrier family 12 member 7 (SLC12A7) expression in vitro. (A) *IGF1* expression levels were measured in adrenocortical carcinoma (ACC) lines SW-13 and NCI-H295R. SW-13 cells, with low endogenous expression of *SLC12A7*,[17] were shown to have low expression of *IGF1* as well, and NCI-H295R cells with high endogenous expression of *SLC12A7*[17] were shown to have a 93-fold increase in expression of *IGF1*. *IGF1R* expression was also found to be higher in NCI-H295R cells compared with SW-13 cells. SW-13 cells served as reference tissue to calculate relative expression levels. Horizontal bar, mean; error bars, SD.

However, an SLC12A7 transcriptional regulatory role for the IGF system or vice versa, has not been reported in ACC. To explore the possibility of an IGF signaling-mediated paracrine/autocrine loop for modulating SLC12A7 expression, and thereby a more aggressive behavior in adrenal cancer, ACC cell line SW-13 with low endogenous SLC12A7 expression[17] was treated with recombinant IGF1 and IGF2 for varying time intervals. No significant effect was seen on *SLC12A7* mRNA expression (data not shown) by exogenous IGF1/2 treatments. On the other hand, SW-13 cells with low endogenous expression of *SLC12A7*[17] were shown to have low expression of *IGF1* and *IGF1R*, and NCI-H295R cells, which have relatively high endogenous expression of *SLC12A7*,[17] were shown to have a 93-fold and 13-fold increase in expression of *IGF1* and *IGF1R* (Fig. 5), respectively. In addition, RNA interference silencing of *SLC12A7* in NCI-H295R cells demonstrated a nearly 50% decrease in expression of *IGF1* (Fig. 6A). Western blot analysis also demonstrated decreased protein expression of IGF1 and IGF1R (Fig. 6B) with RNA interference silencing, suggesting an IGF1-SLC12A7 circuitry in ACC development.

Previous studies have demonstrated that IGF1 binding of IGF1R causes IGF1R autophosphorylation and downstream signal transduction, ultimately promoting cellular growth, transformation, and anti-apoptotic properties.[28] To determine whether increased IGF1 expression in ACC promotes IGF1R activation, representative samples were tested for IGF1R phosphorylation using immunohistochemical techniques. As shown in Figures 7 and 8, samples with low IGF1 expression (Figs. 8A, 8B) demonstrated minimal expression of phosphorylated IGF1R, and samples with high IGF1 expression showed increased expression of phosphorylated IGF1R (Figs. 8B, 8C). These results suggest that IGF1 overexpression observed in subsets of ACC tumors can promote tumor formation or progression, in part, through IGF1 specific IGF1R activation.

DISCUSSION

Multiple comprehensive next-generation sequencing studies underscored the diversity of genetic and signaling aberrations, including widely reported IGF dysregulation, in promoting adrenocortical carcinogenesis.[19,20,29] Although *IGF2* overexpression has been previously associated with alterations in gene methylation levels, it has been unclear what changes, if any, affected the *IGF1* locus. Here we show that the *IGF1* gene copy number is significantly affected by gene amplifications in the Yale/Karolinska study cohort, supported by another independent cohort (TCGA) with similar levels of gene copy alterations measured. These findings again highlight that gene CNVs can play a significant role in ACC tumorigenesis and that additional studies are warranted to investigate the potential contributions of CNVs in the global signaling dysregulations generally noted in ACCs.

Recent studies also showed a clear role for dysregulated potassium-chloride channel protein SLC12A7 expression in conferring an aggressive phenotype to ACC.[16,17] However, a potential role for dysregulated IGF signaling in modulating SLC12A7-promoted tumor behavior seen in other cancer types has not been reported in ACC. This potential relationship is investigated in this study using a large cohort of ACC patient samples and 2 widely studied established ACC cell lines. Here we report increased expression of either *IGF1* (16% of samples) or *IGF2* (83% of samples). Although 2 previous studies reported absence of overexpression of *IGF1* mRNA transcripts[23,24] in ACC, we found relatively higher *IGF1* expression in 5 of 33 samples of ACC, similar to an early 1991 study that showed IGF1 overexpression in ACC using immunohistochemical

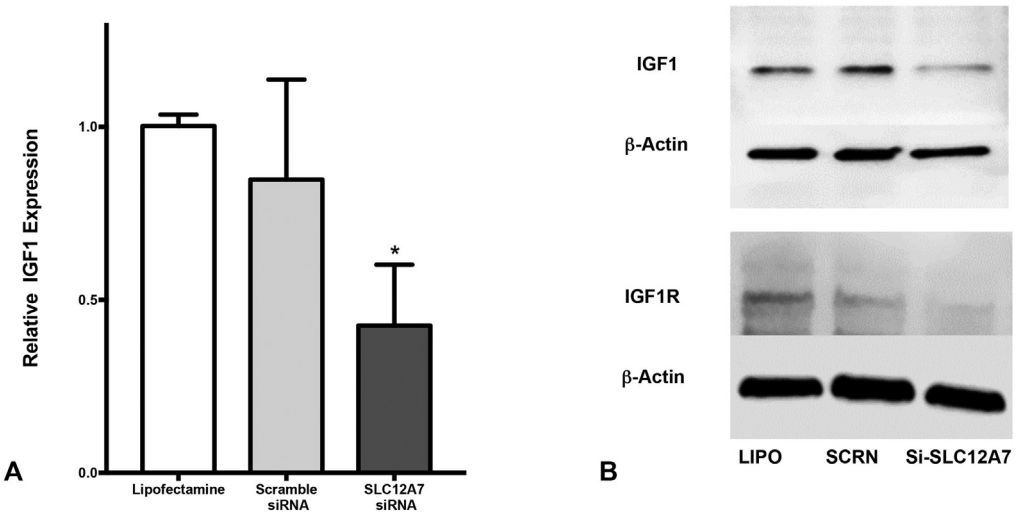

Figure 6. Insulin-like growth factor 1 (IGF1) expression in adrenocortical carcinoma cells silenced for solute carrier family 12 member 7 (SLC12A7) expression. (A) NCI-H295R cells were subjected to *SLC12A7* RNA interference (RNAi) gene silencing, and *IGF1* gene expression levels were measured. *IGF1* expression levels were lower in NCI-H295R cells compared with non-transfected cells (lipofectamine [LIPO] only, $p < 0.05$) and cells transfected with scrambled small interfering RNA ($p = 0.09$). (B) NCI-H295R subjected to SLC12A7 RNAi gene silencing also demonstrated decrease protein expression of IGF1 (upper panel) and IGF1R (lower panel) at 36 and 48 hours post-transfection respectively, as determined by Western immunobloting. β-actin expression is used as protein loading control. Horizontal bar, mean; error bars, SD. SCRN, scrambled RNA.

techniques.[14] The disagreement in IGF1 expression profiles reported by these studies could be related to the quasi-quantitative methods used and/or the relatively low numbers of samples analyzed in those studies. In addition, it remains unclear whether uncharacterized post-transcriptional and/or post-translational mechanisms are also involved in modulating the expression patterns of IGFs in ACC.

The significant association between *IGF1* overexpression and the non-functional status of the tumors found in this study could be associated with SLC12A7 overexpression, a potassium and chloride ion channel. Related ion channel dysregulation has recently been shown to alter hormone secretion in benign adrenal tumors,[30,31] however, additional investigation would be needed to determine the potential causal relationship observed in malignant ACCs. In addition, analogous relationships between IGF1 signaling and SLC12A7 has been reported previously in multiple cancer types.[18,32]

Previous studies have also demonstrated that IGF1 stimulates recruitment of SLC12A7 from the cytosolic pool to the cell membrane with associated increase in activity of

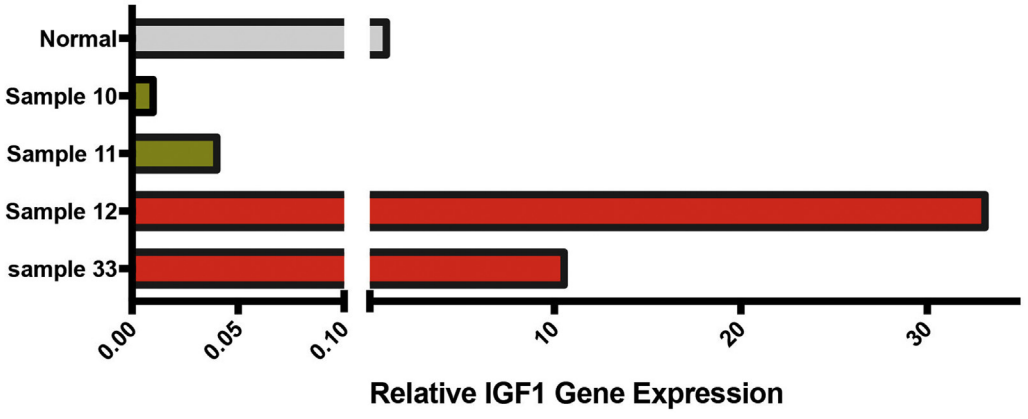

Figure 7. Insulin-like growth factor 1 (IGF1) expression of selected adrenocortical carcinoma samples. Samples 10 and 11 have relatively low expression of *IGF1* compared with normal adrenal tissue, and samples 12 and 33 both have a more than 10-fold increase in expression.

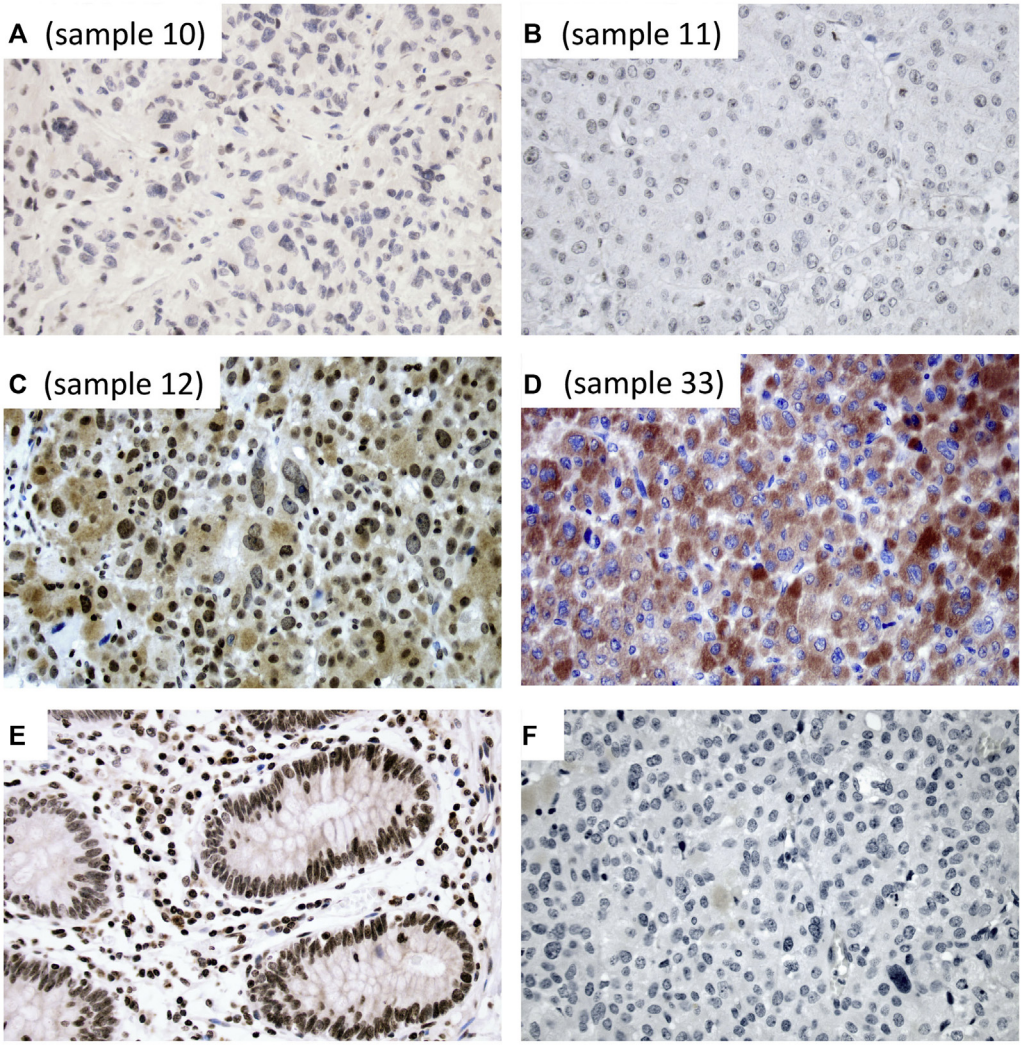

Figure 8. Insulin-like growth factor 1 receptor (phosphoY1161) immunoreactivity in adrenocortical carcinoma (ACC) samples. All photomicrographs are magnified ×400. (A, B) ACC cases 10 and 11, respectively, displaying negative immunoreactivity. (C) ACC case 12 displaying regional nuclear and cytoplasmic immunoreactivity, and other areas of the same tumor displayed only weak expression (data not shown). (D) ACC case 33 displaying cytoplasmic immunoreactivity and absence of nuclear staining. This pattern was heterogeneous, with adjacent areas displaying negative staining (data not shown). (E) Positive control (colon mucosa). (F) Omission of the primary antibody for each tissue (as represented here for ACC case 1559) served as negative control.

KCC related ion channels, including SLC12A7.[32] The IGF stimulation alone might not be the consummate cause of SLC12A7 overexpression in ACC, but can support its function by facilitating its transportation at the cell membrane. Indeed, it has been shown previously that ACC cells overexpressing SLC12A7 localize preferentially at the leading edge of cell extensions.[17]

Similar to previous reports, we have shown overexpression of *IGF2* in nearly all tumors tested, which has been consistently shown to be a molecular hallmark of ACC.[9] Comprehensive genetic analyses also have shown *IGF2* overexpression to be present in all genetic subtypes of ACC,[20] possibly indicating that IGF2 signally does not represent a unique molecular signature that applies to a subset of adrenal tumors, but rather a global signaling event complementing a variety of driving events in adrenal tumorigenesis, such as *CTNNB1* and *TP53* mutations. Although we observed higher *IGF2* expression in larger tumors, it did not portend any effect on survival. This could be due, in part, to the limited number of stage I tumors in our cohort, or the mere bulk of the tumor tissue synchronously producing IGFs.

Despite a plethora of in vitro and in vivo data indicating the IGF system to be a promising target in ACC

treatment, a recent phase 3 clinical trial inhibiting IGF1R signaling with linsitinib (OSI-906) failed to show a survival benefit compared with the placebo control, and was stopped early because of a lack of response.[33] However, an earlier study targeting IGF1R and mammalian target of rapamycin simultaneously demonstrated stable disease in 11 of 26 patients.[34] These findings suggest that targeting IGF signaling alone might not be sufficient for improving treatment outcomes, but could prove to be helpful in combination with other targets, such as dysregulated ion channels, as suggested in this study.

CONCLUSIONS

Here we show that increased IGF1 stimulation is correlated with SLC12A7 overexpression in non-functional, early-stage ACCs, suggesting a targeted SLC12A7/IGF1 therapeutic opportunity for the subset of ACCs profiled for their co-expression.

Author Contributions

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Analysis and interpretation of data: Brown, Nicolson, Stenman, Juhlin, Korah, Carling

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