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Recurrent Amplification of the Osmotic Stress Transcription Factor *NFAT5* in Adrenocortical Carcinoma

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Tumorigenesis requires mitigation of osmotic stress and the transcription factor nuclear factor of activated T cells 5 (NFAT5) coordinates this response by inducing transcellular transport of ions and osmolytes. NFAT5 modulates in vitro behavior in several cancer types, but a potential role of NFAT5 in adrenocortical carcinoma (ACC) has not been studied. A discovery cohort of 28 ACCs was selected for analysis. Coverage depth analysis of whole-exome sequencing reads assessed *NFAT5* copy number alterations in 19 ACCs. Quantitative real-time PCR measured *NFAT5* mRNA expression levels in 11 ACCs and 23 adrenocortical adenomas. Immunohistochemistry investigated protein expression in representative adrenal samples. The Cancer Genome Atlas database was analyzed to corroborate *NFAT5* findings from the discovery cohort and to test whether *NFAT5* expression correlated with ion/osmolyte channel and regulatory protein expression patterns in ACC. *NFAT5* was amplified in 10 ACCs (52.6%) and clustered in the top 6% of all amplified genes. mRNA expression levels were 5-fold higher compared with adrenocortical adenomas ($P < 0.0001$) and *NFAT5* overexpression had a sensitivity and specificity of 81.8% and 82.7%, respectively, for malignancy. Increased protein expression and nuclear localization occurred in representative ACCs. The Cancer Genome Atlas analysis demonstrated concomitant *NFAT5* amplification and overexpression ($P < 0.0001$) that correlated with increased expression of sodium/myo-inositol transporter *SLC5A3* ($r^2 = 0.237$, $P < 0.0001$) and 14 other regulatory proteins ($P < 0.05$) previously shown to interact with NFAT5. Amplification and overexpression of *NFAT5* and associated osmotic stress response related genes may play an important role adrenocortical tumorigenesis.

Key Words: Adrenocortical carcinoma, NFAT5, Osmotic Stress, and SLC5A3

Adrenocortical carcinoma (ACC) is a rare malignancy, with an average annual incidence recently estimated to be 1.02 cases per million people [1]. Patients typically present in the fourth or fifth decades of life with symptoms resulting from local mass effect and/or hormone overproduction. En bloc, R0 surgical resection remains the primary treatment modality and provides the only opportunity to obtain cure for patients with localized tumors [2]. Unfortunately, many patients present with metastatic disease, locally advanced and/or

Abbreviations: ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; CNA, copy number alteration; NFAT5, nuclear factor of activated T cells 5; RNA-Seq, RNA sequencing; TCGA, The Cancer Genome Atlas; WES, whole-exome sequencing.

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unresectable disease, or shortly develop recurrence after surgical treatment, despite R0 resection [3]. For patients with metastatic disease, the 5-year survival was recently estimated to be less than 50% based on analysis of the national cancer database [4], but has been reported to be as low as 15% in some studies [5]. Therefore, improvements in treatment options are greatly needed.

Several types of systemic therapies are available for adjuvant therapy or treatment of advanced and metastatic disease. For several decades, the adrenolytic medication mitotane has served as the primary treatment regimen and has been shown to reduce recurrence [6] and improve survival [7], though its response can be heterogeneous and some studies have not shown a clear benefit [8]. It remains uncertain which patients benefit most from Mitotane, as those patients with low-grade, early-stage tumors may not need to be treated [9]. Furthermore, the initiation, maintenance, and tolerance of mitotane therapy can be limited by its challenging pharmacokinetic profile and associated drug toxicity [10].

Cytotoxic agents have also been used in the treatment of ACC, classically with streptozotocin. In 2012, a clinical trial reported the use of combination chemotherapy with etoposide, doxorubicin, and cisplatin with mitotane to be superior to streptozotocin and mitotane in patients with metastatic disease. That said, only 23% of patients responded to the etoposide, doxorubicin, and cisplatin with mitotane regimen [11]. In addition to chemotherapy regimens, targeted and immune therapies have been investigated in preclinical and clinical trials, including insulin growth factor, mTOR, tyrosine kinase, and immune-checkpoint inhibitors. Some studies have shown benefit, though additional studies are needed [9].

Although incremental improvements have been made for subsets of ACCs [5], the overall prognosis for patients remains poor. Current evidence suggests better outcomes are likely to be achieved with combination therapies that simultaneously target multiple pathways involved in adrenal tumorigenesis [12, 13]. This is supported, in part, by recent genomic studies demonstrating many cancer-associated pathways to be affected in ACCs in the general setting of genomic instability highlighted by frequent and large gene copy number alterations (CNAs) [14-16].

As such, it is critical to discover as many relevant pathways involved in adrenal tumorigenesis. Previous studies have shown that mitigation of osmotic stress is important for cancer development and progression. Modulation of osmotic forces plays an essential role in cancer survival, growth, and metastasis [17, 18], though very little is known about this in ACC. We recently showed that nuclear factor of activated T cells 5 (NFAT5) overexpression was associated with in vitro metastatic behavior in ACC cell lines SW-13 and NCI-H295R [19]. NFAT5 is an established osmotic-stress transcription factor and coordinates osmotic stress responses by inducing expression of transmembrane transporters of ions and osmolytes [20]. NFAT5 has been implicated to modulate the malignant behaviors of other cancer types in processes including angiogenesis, invasion, glycolysis, and osmotic stress regulation [21-26]. A potential role for *NFAT5* in adrenocortical tumorigenesis is largely unexplored, and is investigated here.

1. Material and Methods

A. Study Cohort

Following approval by the Yale and Karolinska Institutet institutional review boards, 28 cases of histologically confirmed ACCs and 23 cases of histologically confirmed adrenocortical adenomas (ACAs) were selected for molecular and clinical analysis (Yale-Karolinska cohort). Protection of human subjects in the publicly available The Cancer Genome Atlas (TCGA) database (n = 92) was described in its associated publication [16]. Patient demographic and clinical characteristics of the Yale-Karolinska cohort are shown in Table 1. Fresh-frozen adrenal tissue samples were prospectively maintained in endocrine tumor repositories and experienced endocrine pathologists reviewed tissue sections for confirmation of the diagnosis

before the investigation. Because of the rarity of the ACCs, some samples were only available in archived formalin-fixed, paraffin-embedded form and thus were not subjected to gene expression analysis.

B . Tumor Gene Copy Number Analysis

ACC samples were previously subjected to whole-exome sequencing (WES) and chromosomal arm level CNAs were reported by Juhlin et al [15]. A follow-up analysis was performed to investigate single-gene CNAs of the *NFAT5* locus on chromosome 16q in 19 samples. Gene copy number was determined by assessing the ratio of coverage depth of WES reads between tumor and adjacent normal adrenal DNA. Univariate statistical analysis and Genomic Identification of Significant Targets in Cancer version 2.0 testing determined the significance of *NFAT5* CNAs. Considering tumor impurity from normal diploid cells, $\log_2$ transformation of tumor/normal WES read ratios of < -0.3, -0.3 to 0.3, and > 0.3 was used to delineate loss, no change, and amplification of gene material, respectively. *NFAT5* CNAs in the exploratory cohort were compared with a larger, confirmatory cohort from TCGA database using the Xena platform (UC Santa Cruz) of ACC CNAs [16].

C. Tumor Gene Expression Analysis

RNA was isolated from fresh-frozen samples using the RNeasy Plus Mini Kit (Qiagen). Quantity and quality of isolated RNA was determined by spectrophotometry (NanoDrop Technologies) and 200 ng of RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR 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 *NFAT5 (Hs00232437_ml)* and the housekeeping gene large ribosomal protein 0 (*RPLP0; Hs00420895_gH*). Relative expression levels were calculated using the Livak method [27]. The normal reference tissue analyzed in this study

Table 1. Demographics and Clinical Characteristics

	ACC	ACA
Total number	28	23
Yale (%)	14 (50.0)	23 (100.0)
Karolinska (%)	14 (50.0)	0 (0)
Gender		
Male (%)	8 (28.6)	5 (21.7)
Female (%)	20 (71.4)	18 (78.3)
Age (years)		
Mean ± SD	56.9 ± 12.8	48.3 ± 12.2
Range	28 - 77	28 - 74
Size (cm)		
Mean ± SD	11.8 ± 4.4	2.9 ± 1.6
Range	5.5 - 21.0	1.1 - 6.5
Hormone		
Aldosterone	1	9
Cortisol	9	7
Androgen	5	0
Multisecreting	5	0
Nonfunctional	8	7
ENSAT		
I	0	NA
II	12	NA
III	9	NA
IV	7	NA

ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; cm, centimeter; ENSAT, European Network for the Study of Adrenal Tumors; SD, standard deviation.

for comparison included 12 samples of histologically normal adrenal tissue surgically removed along with adjacent adrenal hyperplasia/adenoma samples. Real-time quantitative PCR assays were performed in duplicates or triplicates. In total, 11 ACCs and 23 ACAs were tested.

The TCGA database of ACC *NFAT5* RNA sequencing reads (RNA-Seq) was queried via the Xena platform (https://xenabrowser.net, UC Santa Cruz) to analyze the potential association between *NFAT5* CNAs and expression levels. Similarly, known targets of NFAT5 transcription factor activity, including *AKR1B1*, *SLC5A3*, *SLC6A6*, *SLC6A12*, and *PNPLA6* [20], as well as regulatory proteins that interact with NFAT5 [28], were assessed for correlating *NFAT5* expression patterns in the TCGA database using the Xena platform.

D. Immunohistochemistry

Five-micrometer-thick representative sections of histologically confirmed ACCs, ACAs, and normal adrenal tissue from archived formalin-fixed, paraffin-embedded pathology samples were selected for study. With the use of standard immunohistochemistry protocols, target epitopes were detected with rabbit anti-NFAT5 polyclonal antibody (Invitrogen, catalog #PA1-023, RRID: AB_2152617) [29] followed by goat anti-rabbit HRP conjugated monoclonal secondary antibody (Invitrogen, catalog #A16104, RRID:AB_2534776) [30]. 3,39-diaminobenzidine tetrahydrochloride was used for antigen detection (Life Technologies). Sections were counterstained with hematoxylin and eosin and mounted using ImmunoHistoMount (Santa Cruz Biotechnology). Images were acquired at 100× and 400×.

E. Statistics

A univariate analysis was performed. A 1-sample *t*-test was used to determine the significance of gene copy alterations. A 2-tailed Welch's *t*-test or Mann-Whitney test was used to assess differences in 2 groups with continuous distribution, for normal and non-normal variables, respectively. For variables with greater than 2 dependent values, ANOVA was used. Pearson correlation was used to compare matched continuous variables. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated using a relative gene expression of 1.0 to delineate under and overexpression of *NFAT5*. Survival data were assessed by Kaplan-Meier methods and differences were compared by the Mantel-Cox test. Statistical analyses were performed using GraphPad Prism 7 and Xena platform associated statistical analyses. Visualization of putative protein interactions was performed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 11.0 [31]. A P value < 0.05 was considered statistically significant.

2. Results

Nineteen samples were analyzed for *NFAT5* CNAs in the Yale-Karolinska cohort. Overall, *NFAT5* ranked in the top 6% of all genes amplified, thus representing a locus highly involved in gene amplifications compared with other loci. In particular, amplifications were observed in 10 samples (52.6%), whereas 5 samples demonstrated heterozygous deletions, and four samples were unaltered by CNAs. Overall, *NFAT5* gene copy alterations deviated from normal diploid copy number ($P = 0.0068$, Fig. 1A). As previously reported, chromosome 16q was shown by Genomic Identification of Significant Targets in Cancer version 2.0 to be significantly affected by gene amplifications [15]. Analysis of the TCGA ACC database demonstrated a similar amplification pattern, with 43 of 90 samples (47.8%) showing gene copy gains that deviated from normal diploid copy number ($P < 0.0001$, Fig. 1B).

Where fresh-frozen tissue was available in the Yale-Karolinska cohort, quantitative real-time PCR determined gene expression levels in 11 ACCs and 23 ACAs. Twelve normal tissue samples served as a reference control. Overall expression levels were approximately 5-fold higher in ACCs compared with ACAs ($P < 0.0001$, Fig. 2). Of the 11 ACCs analyzed, only 3

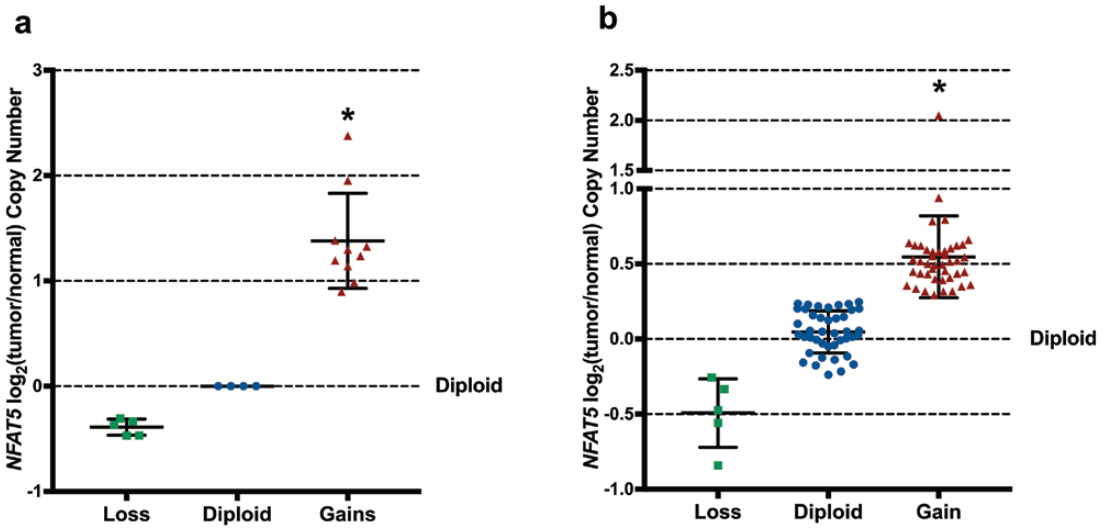

Figure 1. *NFAT5* gene copy analysis. (A) Nineteen samples previously underwent WES (15). Gene copy number was determined by assessing the ratio of coverage depth of WES reads between tumor and adjacent normal adrenal DNA. The *NFAT5* gene was significantly affected, ranking in the top 6% of all amplified genes. Overall, *NFAT5* gene copy alterations significantly deviated from normal diploid copy number (*$P = 0.0068$). (B) Analysis of the TCGA ACC cohort database demonstrated similar gene copy alterations in the *NFAT5* locus (*$P < 0.0001$). NFAT5, nuclear factor of activated T cells 5; TCGA, The Cancer Genome Atlas; WES, whole-exome sequencing. Horizontal bar, mean; error bars, standard deviation.

samples had expression levels lower than the highest expression levels measured in the 23 ACAs tested, thus the expression levels observed between the 2 tumor types were nearly mutually exclusive. The sensitivity, specificity, positive predictive value, and negative predictive value of *NFAT5* overexpression for malignancy of tested adrenal tumors was 81.8%, 82.6%, 69.2%, and 90.5%, respectively. A similar comparison could not be performed in the TCGA database because of the lack of ACA data.

Immunohistochemistry was used to assess protein expression in representative samples of normal adrenal tissue, ACAs, and ACCs. Overall, NFAT5 nuclear and cytoplasmic immunoreactivity was observed to be stronger and more diffuse in ACC compared with normal adrenal and ACA tissues. Moreover, increased nuclear immunoreactivity (right panel, arrows) of NFAT5 protein was predominantly observed in ACCs, suggesting increased transcriptional activity in ACCs compared with normal adrenal and benign tumor tissue (Fig. 3).

Because of limited fresh tissue availability, only 2 ACC samples from the Yale-Karolinska cohort had both corresponding CNA and gene expression data. A diploid sample had an increase fold change expression of 0.73 (moderately overexpressed). The other sample, which was shown to have 2 additional copies (4N), had a fold expression change of 1.58 (moderately to highly overexpressed). To better assess the association between *NFAT5* CNAs and gene expression levels, the TCGA ACC database was queried. When tumors were stratified by *NFAT5* predicted gene copy status (deletion, diploid, amplification), expression levels (RNA-Seq) were tightly correlated with CNAs ($P < 0.0001$, amplification vs. no amplification, Fig. 4). These findings are consistent with previous findings demonstrating gene copy number alterations to affect gene expression levels in ACC [32].

To determine whether *NFAT5* overexpression observed in ACC was potentially related with the expression of other genes known to be associated with NFAT5 transcription factor activity and osmotic stress response, a panel of 5 genes (*AKR1B1*, *SLC5A3*, *SLC6A6*, S*LC6A12*, and *PNPLA6*) was tested for correlating expression levels. As reviewed by Burg et al, these 5 genes have been previously been shown to be targets of NFAT5 transcription activity during the response to osmotic stress [20]. Of the 5 genes analyzed, expression of

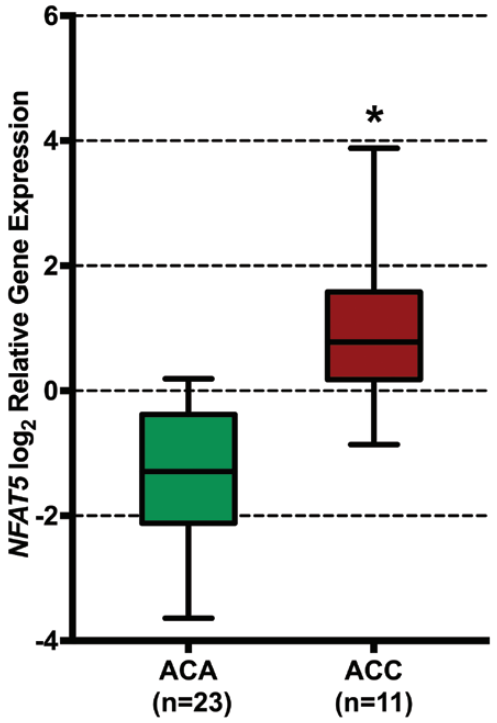

Figure 2. *NFAT5* gene expression analysis. Relative messenger RNA expression levels of *NFAT5* in ACCs (n = 11) were measured by real-time quantitative PCR and compared with expression levels in ACAs (n = 23). Twelve samples of normal adrenal tissue served as a reference control. Overall expression levels were approximately 5-fold higher in ACC compared with ACA (*$P < 0.0001$). ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; NFAT5, nuclear factor of activated T cells 5. Horizontal bar, median; error bars, minimum and maximum.

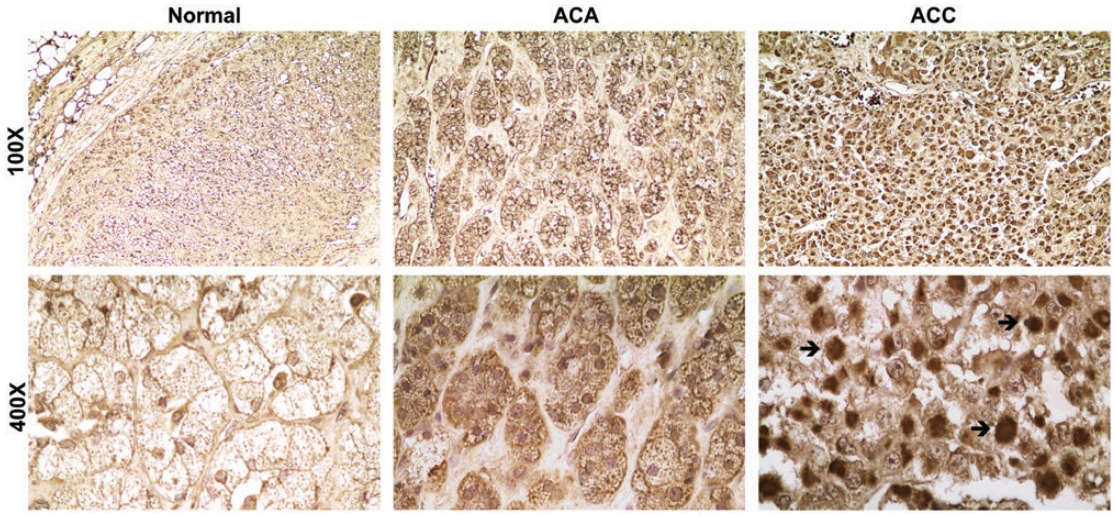

Figure 3. NFAT5 immunohistochemical analysis. Immunostaining of NFAT5 in a representative sample of ACC compared with ACA and normal adrenal tissue. Overall, protein expression levels were higher with increased expression in ACC compared with ACA and normal samples. Furthermore, increased nuclear localization of NFAT5 protein was predominantly observed in ACC samples. Magnification, 100× and 400×; NFAT5 = brown. ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; NFAT5, nuclear factor of activated T cells 5.

SLC5A3 was tightly associated with *NFAT5* expression ($r^2 = 0.2327$, $P < 0.0001$, Fig. 5). SLC5A3 is an ion channel protein that couples sodium transmembrane transport with myo-inositol transport to increase intracellular myo-inositol concentrations in response to osmotic stress [33].

NFAT5 has been previously shown to interact with and/or be regulated by 58 proteins as reviewed by DuMond et al in 2016 [28]. To test which of these proteins may potentially be associated with NFAT5 expression in ACC, RNA-Seq data were queried in the TCGA ACC database for associated expression patterns with *NFAT5* expression levels. Of the 58 genes tested, 14 (Table 2) demonstrated a statistically significant correlation with *NFAT5* expression patterns. Of those 14 genes, 5 (*ATM*, *HNRNPM*, *PARP1*, *PIK3R1*, and *XRCC5*) demonstrated r^2 values > 0.1 (Fig. 6A). Representative imaging of putative protein interactions are demonstrated in Fig. 6B using STRING version 11.0 [31], showing statistically significant interactions (PPI enrichment $P = 6.98e\text{-}7$) among the 14 proteins and NFAT5. It has previously been shown that *NFAT5* is overexpressed with solute carrier

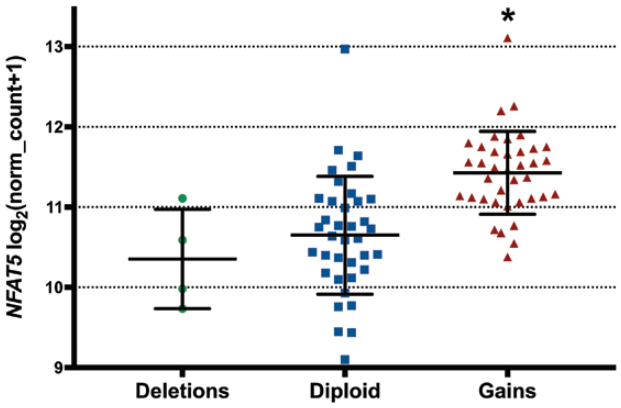

Figure 4. *NFAT5* gene copy gains and expression. When tumors were stratified by *NFAT5* gene copy status (deletions, diploid, amplifications), gene expression levels were tightly correlated with gene copy number (*$P < 0.0001$). *NFAT5*, nuclear factor of activated T cells 5. Horizontal bar, mean; error bars, standard deviation.

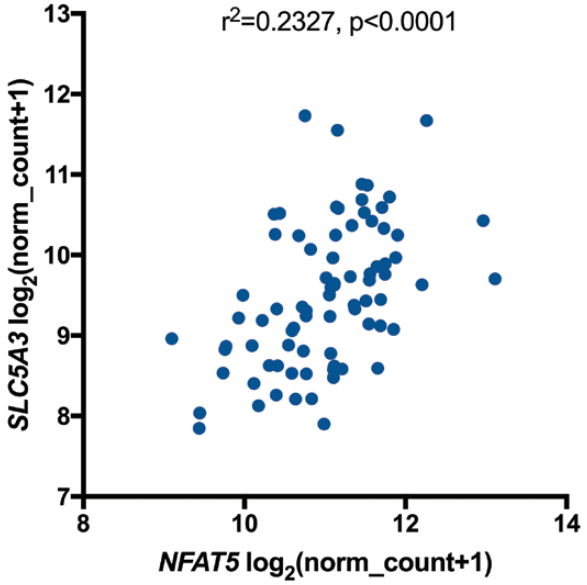

Figure 5. *NFAT5* and *SLC5A3* expression. Messenger RNA expression of *SLC5A3* was associated with *NFAT5* mRNA expression ($r^2 = 0.237$, $P < 0.0001$). NFAT5, nuclear factor of activated T cells 5; SLC5A3, solute carrier family 5 member 3.

Table 2. Genes Coexpressed with *NFAT5* in ACC

Gene	r^2 value	*P* value
AKT1	0.05	0.04
ATM	0.20	0.00
DDX17	0.07	0.02
HNRNPM	0.15	0.00
HSPA8	0.07	0.02
ITGA1	0.06	0.03
JUN	0.06	0.02
MAP2K1	0.10	0.01
MAP2K6	0.06	0.03
MAPK13	0.07	0.02
PARP1	0.12	0.00
PIK3R1	0.20	0.00
PTK2	0.07	0.02
XRCC5	0.11	0.00

ACC, adrenocortical carcinoma; *NFAT5*, *nuclear factor of activated T cells 5.*

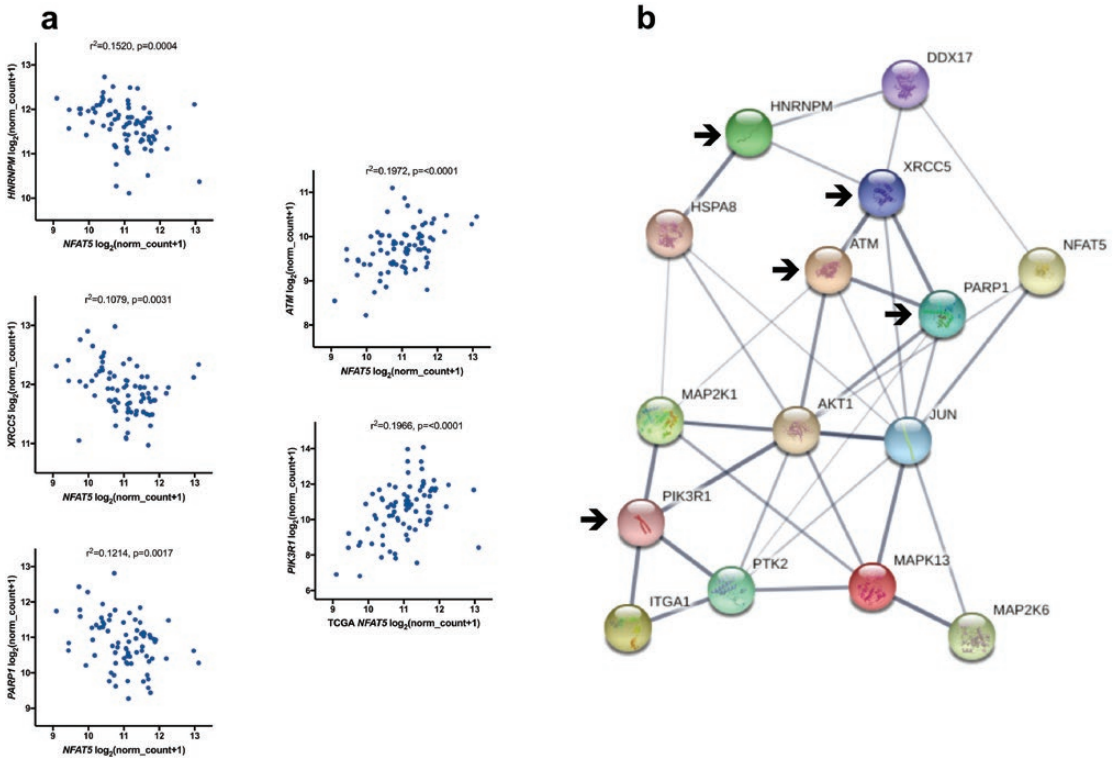

Figure 6. *NFAT5* signaling in adrenocortical carcinoma. (A) Messenger RNA expression levels of 5 representative proteins previously shown to interact with and/or regulate NFAT5 were significantly correlated with *NFAT5* expressions levels ($P < 0.05$). (B) STRING (version 11.0) analysis revealed a statistically significant association of 14 proteins shown in the TCGA cohort to be coexpressed with *NFAT5*. Black arrows mark genes highly correlated with *NFAT5* expression.

SLC12A7 in 2 different ACC cell lines transformed to increase invasion kinetics [19]. Analysis of the TCGA cohort, however, did not show a statistically significant correlation.

NFAT5 gene copy number alterations and expression levels were assessed for correlation with tumor and clinical characteristics in the Yale-Karolinska cohort (Table 3). *NFAT5* copy gains were found to be associated with higher stage tumors (stage I-II vs stage III-IV, $P = 0.0143$), and *NFAT5* gene expression levels were found to be associated

Table 3. Clinical Association with NFAT5 Copy Number and Expression

Tumor Type/Cohort	ACC: Yale-Karolinska		ACC: TCGA		ACA: Yale
***P* Value**	**CNAs**	**Expression**	**CNAs**	**Expression**	**Expression**
Gender	0.63	0.44	0.18	0.14	0.96
Age	0.21	0.87	0.77	0.23	0.84
Size (cm)	0.75	0.56	0.69	0.25	0.17
Hormone	0.53	***0.01***	0.08	0.08	0.71
ENSAT	***0.01***	0.22	0.98	0.95	NA
Survival	0.79	0.43	0.20	0.12	NA

ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; cm, centimeter; CNA, copy number alterations; ENSAT, European Network for the Study of Adrenal Tumors; TCGA, The Cancer Genome Atlas.

with nonfunctional tumors ($P = 0.0121$). To assess these findings in a larger dataset, the TCGA ACC database was analyzed for similar associations. There was a trend of *NFAT5* amplifications and overexpression to be associated with nonfunctional tumors, but a similar association was not seen with stage. An association of *NFAT5* gene copy gains and/or expression levels with survival was not evident in either the Yale-Karolinska or the TCGA cohort.

3. Discussion

The findings reported here identify for the first-time amplification and overexpression of *NFAT5* in ACC. We also observed a close association with *NFAT5* gene amplifications and mRNA overexpression in the TCGA database. Genome-wide CNAs have been previously shown to be a major driver event in ACC with important prognostic significance [16, 34]. Other factors, however, including epigenetic alterations and effects on mRNA stability, might be also affecting *NFAT5* overexpression as well. Indeed, increase sodium concentration has been shown to stabilize *NFAT5* mRNA transcripts, potentially regulated by its 5′ untranslated region, resulting in increased protein production [35].

Although *NFAT5* overexpression is likely caused, in part, by gene amplification, overexpression may also be a consequence of osmotic stress associated with adrenal tumorigenesis, though specific data in ACC are lacking. It has been previously shown that *NFAT5* transcription and nuclear localization is induced by hypertonicity [36, 37]. In cancer, osmotic stress has been shown in colon, renal, and uterine tumor cell line models to induce NFAT5 expression and support survival and growth [23, 26, 38]. Multiple downstream gene targets of NFAT5 have been previously identified in earlier studies. For example, NFAT5 has been shown to up-regulate expression of ion and osmolyte transmembrane protein channels and enzymes in response to osmotic stress [20] and similar examples are observed in cancer cell lines models in the context of mitigating osmotic stress [38, 39]. Here we show that *NFAT5* and *SLC5A3* overexpression patterns were significantly and positively correlated. A similar finding was observed in a uterine tumor cell line [38].

An important finding of this study is the distinctly different expression pattern of *NFAT5* between ACAs and ACCs. In general, ACAs demonstrated suppressed expression, whereas ACCs had increased expression. *NFAT5* mRNA expression patterns demonstrated a relatively high sensitivity and specificity for malignant tumors. The reason for this is not overtly clear, though presumably distinct external biological forces, such as osmotic stress, may be playing an underlying role. Alternatively, as shown by previous studies [15, 40, 41], the overlap between the genetic profile of ACAs and ACCs are minimal and this study further highlights the underlying pathophysiology differences between these 2 entities.

NFAT5 amplifications were associated with higher stage tumors in the Yale-Karolinska cohort. Similar results were observed in pancreatic cancer [24] and glioblastomas [21]. However, a similar result was not seen in the TCGA ACC database, raising the possibility that this positive association is likely related to the bias of the relatively smaller size of the discovery cohort. Although there was a strong association between the malignant status of adrenal tumors and nonfunctional hormonal status, *NFAT5* expression patterns in ACC had limited prognostic properties. This is not surprising. ACC is an extremely heterogeneous cancer type with significant multi-omic alterations [14-16].

In conclusion, *NFAT5* amplification and overexpression is observed in ACCs. *NFAT5* was also coexpressed with other genes associated with the osmotic stress responses. Together, these findings suggest that NFAT5 may play an important role in adrenocortical tumorigenesis.

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Additional Information

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Disclosure Summary: The authors have no conflicts of interest.

Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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