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DNA Copy Amplification and Overexpression of *SLC12A7* in Adrenocortical Carcinoma

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Abstract

Background—Overexpression of *Solute carrier family 12 member 7* (*SLC12A7*) promotes tumor aggressiveness in various cancers. Previous studies have identified the 5p15.33 region, containing the *SLC12A7* locus, frequently amplified in adrenocortical carcinoma (ACC). Copy number amplifications (CNA) may alter gene expression and occur frequently in ACC. However, *SLC12A7* amplifications or expression levels have not been studied in ACC.

Methods—Fifty five cases of clinically well-characterized ACCs were recruited for this study. Whole-exome sequencing (WES) was used to predict CNAs in 19 samples. CNA analysis was performed on an expanded cohort of 26 samples using TaqMan Copy Number Assays. *SLC12A7* mRNA expression was analyzed in 32 samples using real-time quantitative PCR and protein expression was assessed by immunohistochemistry. *SLC12A7* CNAs and expression patterns were evaluated for correlation with patient and tumor characteristics.

Results—WES and Taqman Copy Number Assays demonstrated *SLC12A7* amplifications in 68.4% and 65.4% of ACCs tested, respectively. *SLC12A7* expression levels were increased in ACCs compared to normal adrenal tissue ($p<0.05$). *SLC12A7* overexpression occurred predominantly in samples with amplifications ($p<0.05$), while amplifications were associated with non-functional tumors ($p<0.05$).

Conclusion—*SLC12A7* amplification and overexpression occurs frequently in ACCs and may represent a novel molecular event associated with ACC.

BACKGROUND

Adrenocortical carcinoma (ACC) is a highly aggressive, but rare cancer. Its incidence is estimated to range from 0.7 to 2.0 cases per million people per year. Tumor occurrence is most frequent during the 4th and 5th decades of life, with a higher occurrence observed in women.[1] Risk factors include smoking in men and oral contraceptive use in women.[2]

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Patients typically present because of excessive hormone production, usually from hypercortisolism. However, a significant proportion of patients may present with abdominal and back discomfort from locally advanced cancers. Some patients are incidentally diagnosed by cross-sectional imaging obtained for unrelated reasons.[2] For patients with ENSAT (European Network for the Study of Adrenal Tumors) stage I-III tumors, surgery is the primary treatment modality, with the goal of en bloc, R0 resection. Those patients unable to undergo surgical resection have a significantly worse prognosis. In addition to surgery, external beam radiation, Mitotane, and various chemotherapy regimens are used as either primary therapy for ENSAT stage IV disease or as adjuvant therapy.[3] Despite these aggressive interventions, the overall prognosis for patients remains poor, with reported 5-year survival rates that vary between 37% and 47%.[1]

Several molecular pathways are frequently found abrogated in ACC and likely play a significant role in the genetic origin of ACC. Alteration of the canonical Wnt/β-catenin signaling pathway occurs frequently in ACC via activating mutations of the proto-oncogene *β-catenin*, which stabilizes β-catenin and promotes its translocation to the nucleus. Activated, intra-nuclear β-catenin in turn stimulates target gene transcription and promotes tumor formation.[4] Somatic mutations of the tumor suppressor gene and cell cycle regulator *TP53* occurs in approximately 20–35% of ACCs and are thought to be associated with more aggressive tumors. In addition, Li-Fraumeni Syndrome, which is caused by germline *TP53* mutations, is associated with childhood forms of ACC.[1, 2] One of the most frequently observed molecular events associated with ACC is overexpression of insulin growth factor 2 (IGF2) and its paracrine stimulation of tumor growth. A 100-fold increase in *IGF2* expression levels is observed in 80–90% of tumors.[2] This frequently occurs by either deletion of the imprinted maternal allele with subsequent paternal allele duplication or, less frequently, by loss of maternal allele imprinting.[4]

In addition to these specified pathways, gene copy number variations (CNVs) occur frequently in ACC and are thought to be associated with the malignant behavior of these tumors. Previous studies assessing CNVs in adrenocortical adenomas and ACCs have shown an association between the frequency of CNVs and the malignant status of the tumor (adenoma vs. carcinoma) and tumor size.[5–8] A CNV analysis performed by Stephan *et al.* on 25 ACCs found specific CNV patterns to be associated with survival rates.[9] However, in an analysis utilizing one of the largest cohorts of adrenocortical tumors to study CNVs, only the malignant status of tumors was associated with CNVs. No significant association was observed between CNVs and tumor size or patient survival.[10]

Multiple studies have been performed using various methods and, in general, copy gains are frequently observed on chromosome arms 4q, 4p16, 5p15, 5q12–13, 5q32-qter, 9q34, 12q13, 12q24, and 19 p, while copy losses are observed on chromosome arms 1p, 2q, 11q, 17p, 22p, and 22q.[2] A meta-analysis of CNV studies demonstrated that genes involved in cell cycle regulation, retinoic acid signaling, the complement system, and antigen presentation are frequently affected by copy number changes. However, the same study also demonstrated that only 15% of gene expression alterations observed in ACC could be attributed to CNVs, highlighting the need to validate individual copy alterations with gene expression analyses.[11] In addition, there still remains significant discrepancy among studies identifying genomic

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regions significantly affected by CNVs and the impact of these changes on tumor behavior. Furthermore, until recently, many of these studies utilized methods that identified copy number alterations at chromosome bands. As such, micro-amplifications or micro-deletions involving single genes may not have been identified.

Two recent studies utilized whole-exome sequencing (WES) techniques to clarify and provide an integrated understanding of the molecular pathogenesis of ACC. These studies identified novel genetic mutations previously not associated with ACC tumorigenesis and enabled a more precise, nucleotide level assessment of CNVs. Both studies found the 5p13.33 region to be one of the most amplified regions in ACC.[12, 13] Two interesting genes found in this region include *telomerase reverse transcriptase* (*TERT*) and *Solute carrier family 12 member 7* (*SLC12A7*). *TERT* amplifications and promoter mutations have been previously identified in ACC, though it remains uncertain whether these alterations affect TERT activity and promote tumor malignancy.[12–14] In contrast, the potential, malignancy-promoting role of *SLC12A7* in ACC has not been previously studied.

SLC12A7, a member of the SLC12 gene family, is a 1083 amino acid long, transmembrane protein. Its primary physiologic function is to regulate cell volume via transmembrane potassium and chloride transport.[15] However, recently it has been implicated that SLC12A7 plays a significant role in the development and malignant behavior of several different cancer types. SLC12A7 was frequently found to be overexpressed in cervical, ovarian, and breast cancer tissues. SCL12A7 was also shown to promote *in vitro* and *in vivo* tumor cell growth.[16–19] It was also demonstrated that SLC12A7 co-localizes to the lamellipodia of tumor cells with ezrin, a membrane cytoskeleton linker, and promotes cell migration and invasion.[19] Overexpression of SLC12A7 has been associated with local tumor invasion, lymph node metastases, and poor clinical outcomes in cervical, ovarian, and breast cancers.[15] Based on the previous findings by our group and others that the 5p13.33 region is frequently amplified in ACC, as well as recent studies demonstrating a malignancy promoting role for SLC12A7 in multiple cancers, we aim to investigate whether *SLC12A7* plays a role in adrenocortical carcinogenesis.

METHODS

Study Cohort

All patient healthcare information was acquired and maintained in accordance with regulations as specified by the Health Insurance Portability and Accountability Act (HIPAA). Following approval by Yale University and Karolinska Institutet institutional review boards and informed patient consent, a total of 55 cases of histologically confirmed ACCs were identified for biochemical and clinical analysis. Forty one of those samples recently underwent WES.[13] Because fresh frozen tissue was required for this study, only 32 of the 55 cases were utilized for this investigation. Patient characteristics, including gender, age, tumor size, ENSAT stage, hormone secretory status, and survival were documented following adrenalectomy and is shown in Table 1. All fresh frozen adrenal tissues samples were reviewed by an experienced endocrine pathologist prior to investigation.

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WES Copy Number Prediction

Genomic DNA from 41 ACC samples were recently subjected to whole-exome capture and sequencing as previously described.[13] For this study, single gene CNVs were predicted in 19 of those samples by assessing coverage depth analysis of WES reads between tumor and adjacent normal adrenal DNA.

Quantitative PCR Copy Number Analysis

DNA was isolated from fresh frozen samples using the AllPrep DNA/RNA/Protein Kit (Qiagen). Quantity and quality of isolated DNA was assessed by spectrophotometry (NanoDrop Technologies, Inc). Per the manufactory's instructions, 5 samples yielded inadequate quality and/or concentrations of genomic DNA that precluded further analysis. One sample yielded DNA of sufficient quality and concentration, but the sample failed to amplify despite multiple attempts. As such, a copy number analysis was performed on a total of 26 ACC samples using the TaqMan Copy Number Assays (Applied Biosystems) with primers and probes specific to target gene *SLC12A7* and house-keeping gene *Ribonuclease P RNA Component H1* (*RPPH1*). Normal adrenal tissue was used as a diploid reference control. The assay was performed in quadruplicates. Copy calls were predicted using CopyCaller software v2.0 (Applied Biosystems).

Gene Expression Analysis

RNA was isolated from fresh frozen samples using the AllPrep DNA/RNA/Protein Kit (Qiagen). Quantity and quality of isolated RNA was assessed by spectrophotometry (NanoDrop Technologies, Inc). Two hundred ng of RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR was performed on a CFX96 Real-Time System qPCR machine (Bio-Rad) using TaqMan PCR master mix (Applied Biosystems) with primers and probes specific to *SLC12A7* and housekeeping gene *ribosomal protein large P0* (*RPLP0*). The assay was performed in triplicates. Relative expression levels were calculated using the Livak method (Bio-Rad).

Immunohistochemistry

Five μm-thick representative sections of histologically confirmed ACCs and normal adrenal tissue from formalin fixed paraffin embedded (FFPE) tissue samples were selected for study. Using standard immunohistochemistry protocols[20], target epitopes were detected using rabbit anti-SLC12A7 polyclonal antibody (Origene) followed by goat anti-rabbit HRP conjugated monoclonal secondary antibody (Invitrogen). 3,3'-diaminobenzidine tretrachydrochloride (DAB) was utilized for antigen detection (Life Technologies). Sections were counterstained with hematoxylin and mounted using immunohistomount (Santa Cruz).

Statistical Analysis

The significance of *SLC12A7* CNVs was assessed by the Wilcoxon Signed Rank Test. Continuous variables were assessed for a normal distribution using the D'Agnostino and Pearson omnibus normality test, than analyzed using a 2-tailed *t* test for normally distributed variables, or the Mann-Whitney *U* test for non-normally distributed variables. For variables with greater than 2 dependent values a 1-way analysis of variance and Kruskal-Wallis tests

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were utilized for normally and non-normally distributed populations, respectively. Spearman correlation was utilized to compare matched continuous variable. For categorical variables, the Pearson chi-square test or Fisher's exact test were used, as appropriate. Survival data was assessed by Kaplan-Meier methods and differences were compared by the Mantel-Cox test. A p-value ≤ 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software) and SPSS version 19.0 software (IBM Software)

RESULTS

Copy Number Analysis

As previously described, we and others have shown the 5p15.33 region to be frequently amplified in ACC.[12, 13] To determine which genes were affected by this amplification, a gene-level copy number analysis was performed on 19 samples using WES. The *SLC12A7* locus was predicted to be one of the most amplified loci in ACC with 13 (68.4%) of 19 samples demonstrating copy gains. The remaining samples were predicted to be copy neutral (Table 2). An expanded cohort of 26 ACC samples was subsequently assessed for *SLC12A7* CNVs and compared to normal adrenal tissue using the Taqman Copy Number Assays (Figure 1). In total, 17 of 26 samples (65.4%) demonstrated copy gains, with two samples demonstrating 8 and 9 copies. Eight samples were shown to be copy neutral, and only one sample demonstrated a heterozygous deletion. No homozygous deletions were detected. The overall copy number alterations observed in the ACC samples significantly deviated from the diploid gene copy number observed in normal adrenal tissue ($p<0.05$).

Gene Expression Analysis

SLC12A7 gene expression levels have been shown to be significantly overexpressed in cervical, ovarian, and breast cancers. Furthermore, overexpression of *SLC12A7* was associated with local tumor invasion, lymph node metastases, and poor clinical outcomes in these cancers.[15] As such, gene expression levels of 32 ACC samples were measured and compared to 11 samples of normal adrenal tissue. Overall gene expression levels in the ACC samples were found to be significantly higher than normal adrenal tissue ($p<0.05$, 95% confidence interval 1.69 – 3.84; Figure 2A). Fifteen of 32 samples (47%) demonstrated a 2-fold or greater increase in *SLC12A7* gene expression. In addition, those samples with a 2-fold or greater increase in *SLC12A7* gene expression were associated with ACC samples containing *SLC12A7* gene amplifications ($p<0.05$; Figure 2B). Individual samples analyzed for CNVs and samples with a ≥ 2-fold increase in expression is shown by asterisks in Figure 1.

Immunohistochemistry

To determine SLC12A7 protein expression levels, a representative set of tumor samples were assessed for SLC12A7 protein expression using immunohistochemistry techniques and compared to normal adrenal tissue. Overall expression levels were found to be similar to or greater than expression levels observed in normal adrenal tissue. Furthermore, while staining was observed primarily along membrane compartments in normal adrenal tissue, SLC12A7 staining was also observed in the cytoplasm and nucleus in ACC samples tested (Figure 3).

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Clinical Association

Various clinical parameters (patient gender, age, tumor size ENSAT stage, metastasis, hormone secretory status, and disease-free survival) were assessed for association with *SLC12A7* expression patterns and no significant correlation. *SLC12A7* expression levels tended to be higher in non-functional tumors, potentially suggesting a role in the non-functional phenotype, though that association did not reach statistical significance ($p=0.08$; Figure 3A). In contrast, a statistically significant association was observed between *SLC12A7* gene amplifications and non-functional tumors and male patients ($p<0.05$; Figure 3B&C). No statistically significant association was observed between advanced tumors (ENSAT 3 and 4) and SLC12A7 amplifications.

DISCUSSION

Copy number alterations, including amplifications, have been shown to occur frequently in ACCs in multiple studies using comparative genomic hybridization (CGH) methods. More importantly, the overall frequency of CNVs have been associated with tumor malignancy, size, and survival. These studies have also shown that the 5p chromosome arm is the one most frequent location of genomic amplifications in ACC. However, the genes involved and potentially imparting a more aggressive phenotype by 5p amplifications were not clearly delineated.[5–9] Interestingly, three of these studies also identified low frequency 5p amplifications in adrenal adenomas, indicating that CNVs of chromosome 5 may be an early event in adrenocortical malignancy. [6–8] Two recent studies utilized whole-exome sequencing (WES) techniques to analyze genomic alterations in ACC and both studies found the 5p13.33 region to be one of the most amplified regions in two independent cohorts of ACC patients.[12, 13]

A meta-analysis of CNV studies demonstrated that only 15% of gene expression alterations observed in ACC could be attributed to copy number gains or losses.[11] A more recent study demonstrated that genes in gained loci only had a 1.1 fold increase in overall expression levels, while genes with lost copies only had a 0.9 fold decrease in overall expression levels. [10] These findings demonstrate that the majority of copy number alterations likely represent passenger events and highlights the need to validate potential functional consequences of CNVs with gene expression analyses. Although marginal alterations of expression levels can have functional consequences in gene function, in this study we considered a very stringent, 2-fold increase as a standard for functionally interpretable increase in expression. Using this criteria, 47% of samples showed a significant increase in *SLC12A7* gene expression levels, which was associated with copy gains. Thus, these findings possibly indicate that *SLC12A7* amplifications in ACC may not represent a passenger event.

Multiple transciptome studies have demonstrated significant alterations of transcription levels differentiating ACCs from normal adrenal tissue and benign adrenal adenomas. Furthermore transcriptome analyses have been shown to stratify tumors by prognosis and may also serve as a more reliable predictor of tumor aggressiveness than other commonly used markers, including the Weiss scoring system.[21–23] In this study we observed a nearly 3-fold increase in overall expression levels of *SLC12A7* compared to normal adrenal tissue. Previous studies have demonstrated an association between SLC12A7 overexpression and

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local tumor invasion, lymph node metastases, and poor clinical outcomes in cervical, ovarian, and breast cancers.[15] In this study, however, we did not observe these same associations. With only 32 samples, our analysis may have been under-powered to detect the effects of *SLC12A7* overexpression on disease characteristics and outcomes. In addition, only 21.9% of the study cohort included samples from patients with ENSAT IV disease, limiting our ability to assess the effects of SLC12A7 overexpression potentially imparts on ACC. Furthermore, ACC is a very heterogeneous tumor and numerous molecular aberrations are observed per tumor, potentially obscuring the effects of *SLC12A7* gene copy gains and overexpression. [12, 13]

We did, however, note that *SLC12A7* expression levels tended to be higher in nonfunctional tumors, though this did not reach statistical significance. This is an interesting finding since ion channels have been shown to be critical regulators of adrenal hormone secretion. [24, 25] Similarly, *SLC12A7* amplifications were associated with non-functional tumors. Since gene amplifications typically do not occur in benign adrenal adenomas, *SLC12A7* copy gains may serve as a putative molecular marker of malignancy in indeterminate adrenal tumors, as well as a marker of non-functional tumors.

Immunohistochemical analysis demonstrated increased expression of SLC12A7 in ACC samples compared to normal adrenal tissue. In addition, while staining was observed primarily along the membrane compartments in normal adrenocortical cells, in ACC samples, SLC12A7 staining was also observed in the cytoplasm and nucleus. Previous studies have shown that SLC12A7 is frequently upregulated from an inactive cytoplasmic pool to the cell membrane in various cancer cell lines. This process has been shown to be robustly promoted by insulin growth factor 1 (IGF1) stimulation. While IGF1 is not reported to be overexpressed in ACC, *IGF2* is highly overexpressed in ACC and mitigates its malignancy promoting activity via the IGF1 receptor.[4] Though highly speculative, IGF2 stimulation may represent a novel mechanism promoting SLC12A7 overexpression in ACC.

In conclusion, this study indicates that the *SLC12A7* gene is frequently amplified in ACC, which is associated with significant mRNA and protein overexpression. Furthermore, *SLC12A7* amplifications are associated with non-functional tumors. Future studies will need to assess the functional consequence(s) of *SLC12A7* overexpression in ACC, as well as its use as a potential therapeutic target.

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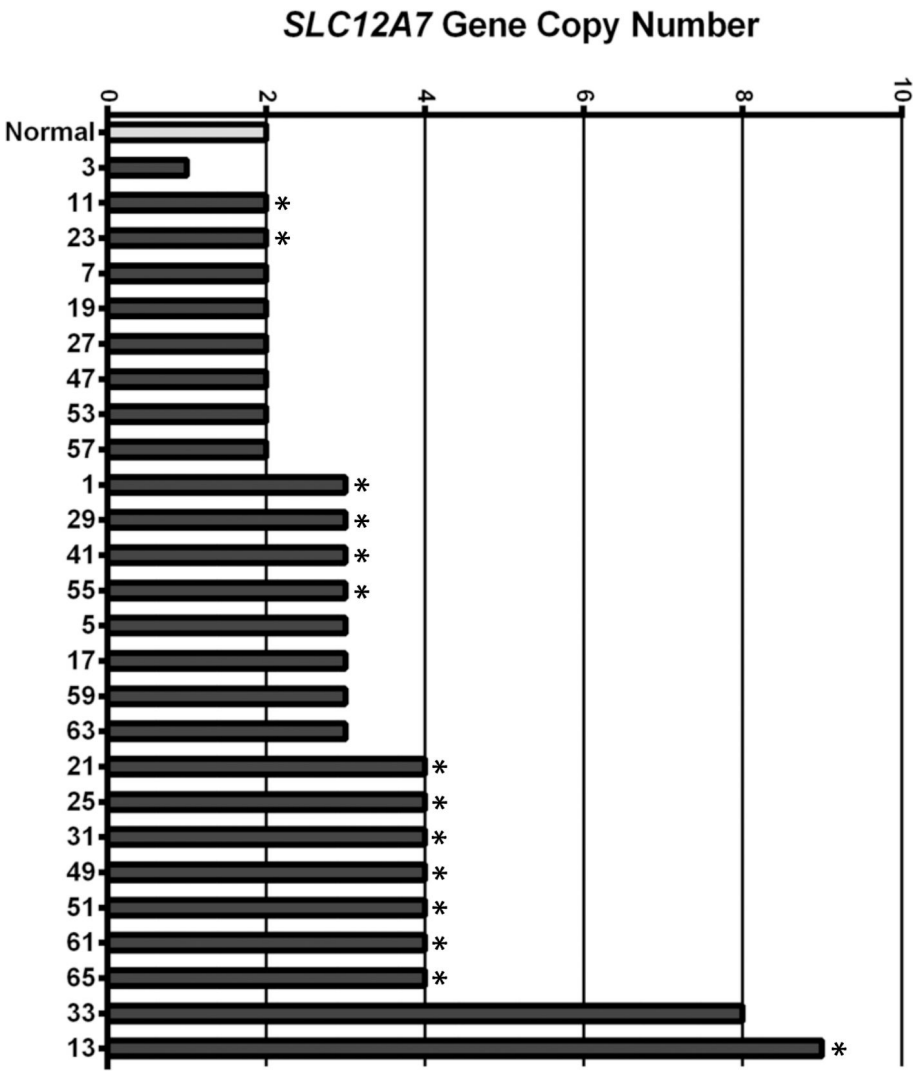

Figure 1: *SLC12A7* gene copy analysis.

SLC12A7 gene copy numbers in adrenocortical carcinomas were measured in 27 samples by TaqMan Copy Number Assays and compared to normal adrenal tissue. *SLC12A7* gene copy number alterations significantly deviated from diploid gene copy numbers observed in normal adrenal tissue ($p<0.05$, Wilcoxon Signed Rank Test). Two gene copies equals diploid. *, samples with ≥ 2-fold increase in *SLC12A7* gene expression. Abbreviations: *SLC12A7, Solute carrier family 12 member 7.*

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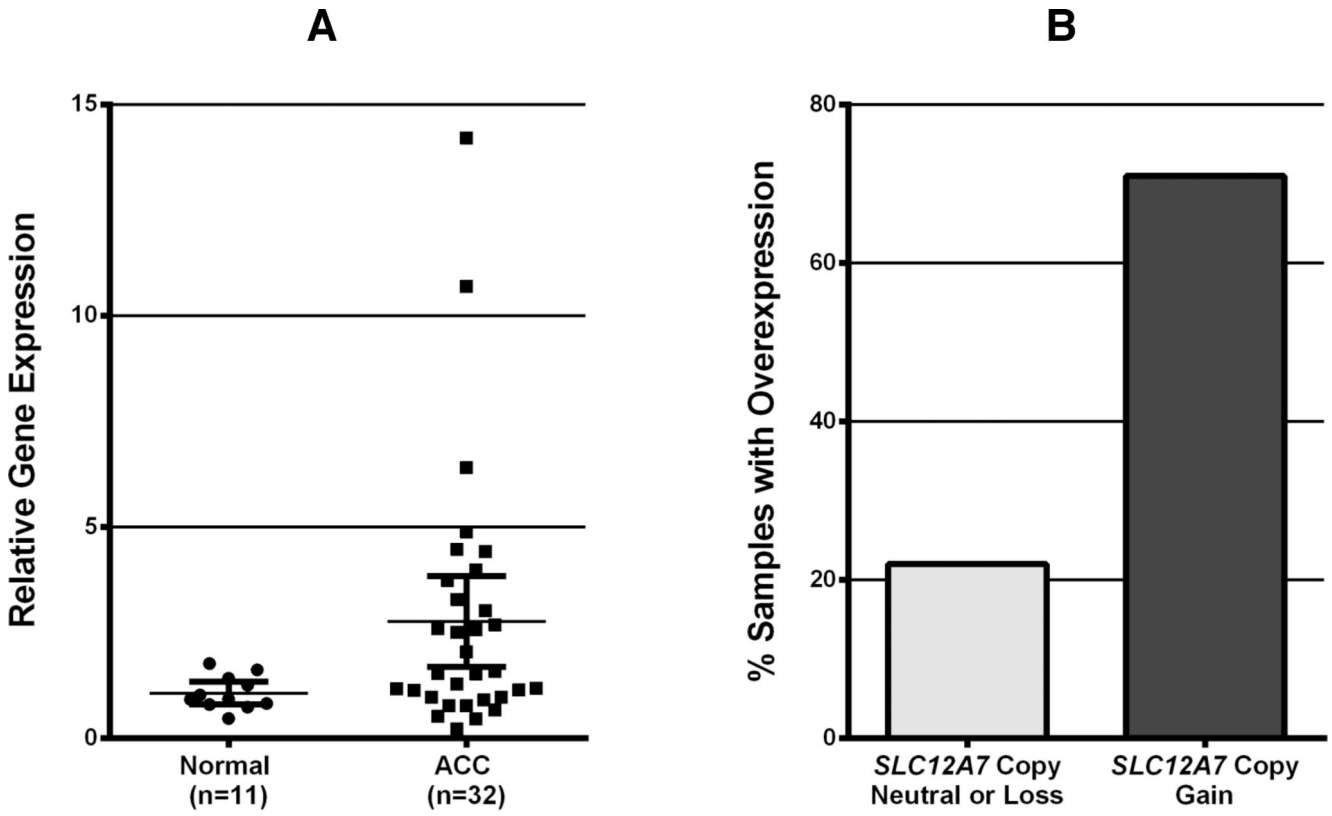

Figure 2: *SLC12A7* gene expression analysis.

A Relative messenger RNA expression levels in adrenocortical carcinoma (ACC) samples (n=32) were measured by real-time quantitative polymerase chain reaction and compared with expression levels in normal adrenal tissues (n=11). Expression values were higher in ACCs compared to normal adrenal tissue ($p<0.05$, Mann-Whitney U test). *Horizontal bar*, mean; *Error bars*, 95% confidence interval. **B** Overexpression of *SLC12A7* is associated with ACC samples containing *SLC12A7* gene copy gains ($p<0.05$, Fisher's exact test). Abbreviations: *ACC*, adrenocortical carcinoma; *SLC12A7*, *Solute carrier family 12 member 7*.

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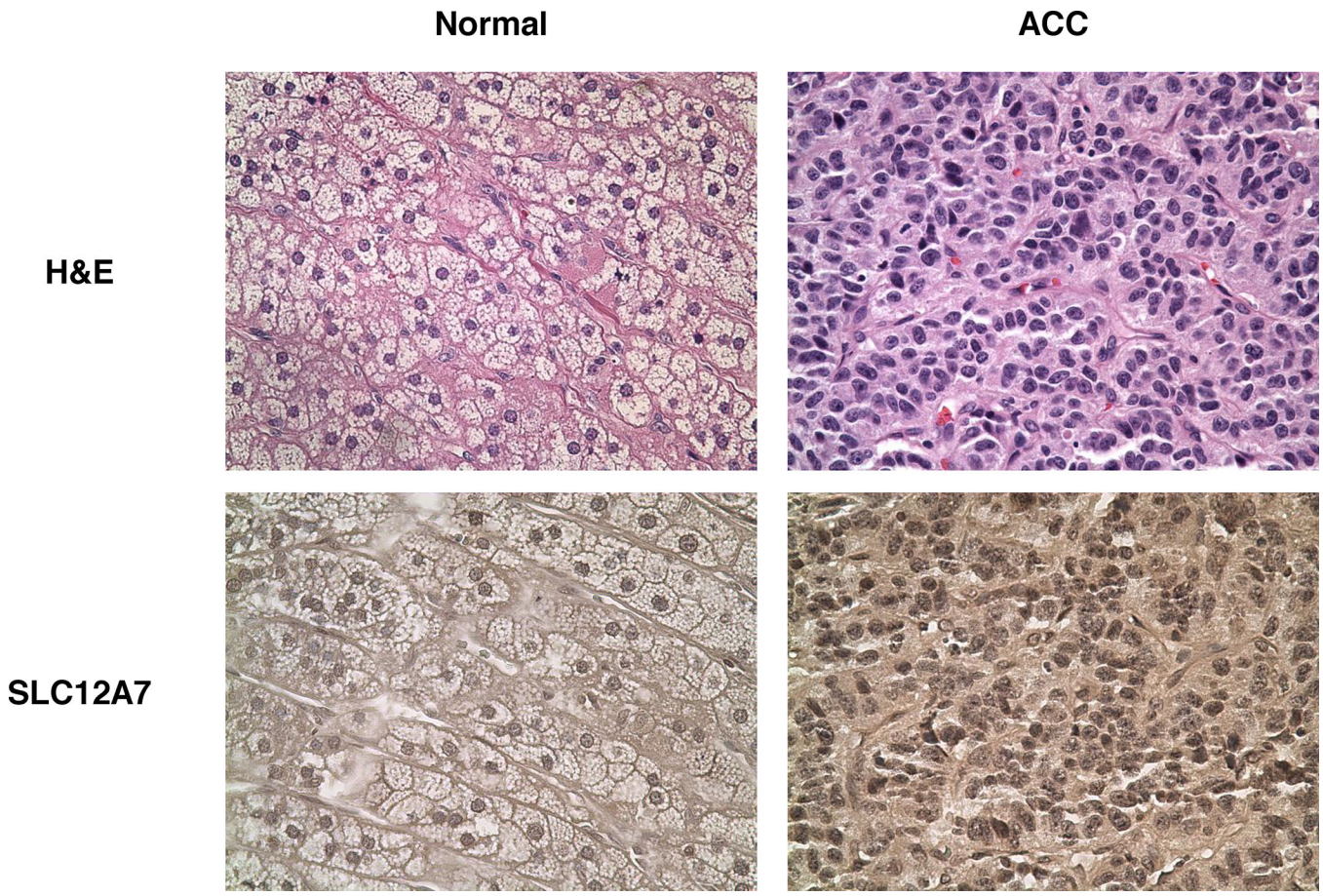

Figure 3: SLC12A7 Immunohistochemical analysis.

Immunostaining of SLC12A7 in a representative sample of adrenocortical carcinoma compared to normal adrenal tissue. Original magnification, X400; SLC12A7 = *brown*).

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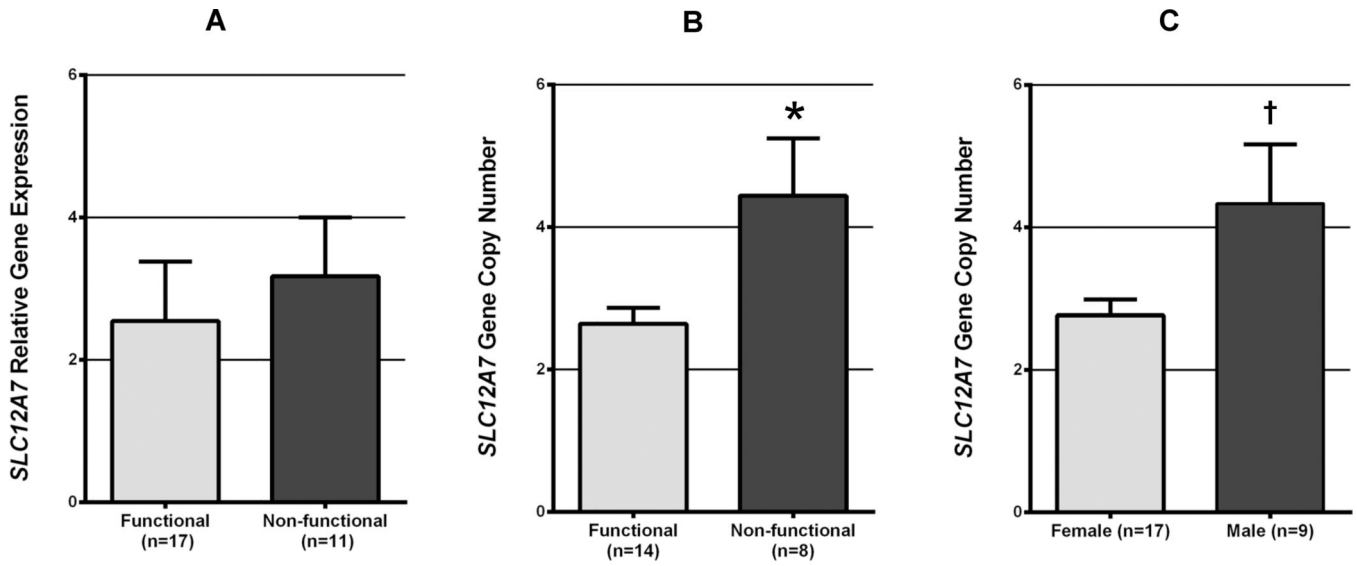

Figure 4: Clinical Analysis.

Various clinical parameters (patient gender, age, tumor size ENSAT stage, metastasis, hormone secretory status, and disease-free survival) were assessed for association with *SLC12A7* expression patterns and amplifications. **A** Higher expression levels was observed in non-functional tumors, but the difference did not reach statistical significance (p=0.08). **B & C** *SLC12A7* amplifications were higher in non-functional tumors (*, $p<0.05$, 2-tailed *t* test), as well as male patients (†, $p<0.05$, 2-tailed *t* test). *Horizontal bar*, mean; *Error bars*, SEM.

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Table 1:

Demographic, staging, and patient outcome information.

Characteristics	Number of Cases	Percentage
Total Number	32	NA
Gender		
Male	12	37.5%
Female	20	62.5%
Age ± SD (y)	57.7 ± 14.0	NA
Cohort		
Yale	7	21.9%
Karolinska	25	78.1%
Tumor Size (cm)		
Mean ± SD	13.0 ± 4.4	NA
Range	5.5 – 21.0	NA
ENSAT 2008 Stage		
I	0	0.0%
II	16	50.0%
III	9	28.1%
IV	7	21.9%
Metastasis at		
Presentation	7	21.9%
Hormone Hypersecretion		
Aldosterone	1	3.1%
Cortisol	8	25.0%
Androgen	3	9.4%
Multi-secreting *	5	15.6%
Non-functional	11	34.4%
No information available	4	12.5%
Outcome		
Alive, no recurrence	9	28.1%
Alive, recurrent	3	9.4%
Death from disease	16	50.0%
Death from other causes	4	12.5%

cm, centimeter; *DHEA,* dehydroepiandrosterone; *ENSAT,* European Network for the Study of Adrenal Tumors; SD, standard deviation; y, years.

*Tumors secreting two or more of the following hormones: aldosterone, cortisol, testosterone, or DHEA.

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Table 2:

SLC12A7 Copy number alterations assessed by whole-exome sequencing and Applied Biosystems TaqMan Copy Number Assays.

Method	# Samples Tested	# Copy Gains	% Copy Gains	# Copy Loss	% Copy Loss
WES	19	13	68.4%	0	0%
AB Taqman	26	17	65.4%	1	3.8%

AB Taqman, Applied Biosystems Taqman Copy Number Assays; *WES,* Whole-exome sequencing.