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“IDENTIFYING MOLECULAR DRIVER EVENTS IN SALIVARY ACINIC CELL CARCINOMAS”

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CERTIFICATE OF APPROVAL

MASTER'S THESIS

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2020

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ABSTRACT

IDENTIFYING MOLECULAR DRIVER EVENTS IN SALIVARY ACINIC CELL CARCINOMAS
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M.S., ORAL PATHOLOGY, 2020

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Introduction: Salivary acinic cell carcinoma (AcCC) is typically a low-grade adenocarcinoma found most commonly in the parotid gland. These tumors can undergo high grade transformation (HGT) and loco-regional recurrence and metastases. Histopathology of this tumor is characterized by acinar differentiation. This histopathology suggests conserved genetic driver events which immortalize cells. Recently NR4A3 has been reported as an oncogenic transcription factor in AcCC. Objective: The purpose of this study is to determine and validate NR4A3 as an oncogenic driver event in AcCC. Methods: Eight cases of AcCC from Walter Reed National Naval Medical Center (WRNNMC) and seven normal parotid gland cases from National Institutes of Health (NIH) were used in this study. RNA and DNA extraction was performed using Qiagen AllPrep DNA/RNA Kits. RNA and DNA samples were assessed for quantity and quality using the Agilent Fragment Analyzer Capillary Electrophoresis System and National Institute of Bioinformatics and Scientific Programming Core. RNAseq data will be analyzed using ChemPipe algorithim detection of chimeric reads and gene fusions. Fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) will be used to further validate the results of this study. Results: DNA and RNA are still being analyzed for quality at the National Institute of Bioinformatics and Scientific Programming Core. Immunohistochemistry for NR4A3 needs to be repeated. FISH studies showed split signals of the NR4A3 gene locus in

four out of five cases. This confirmed a break in the chromosomal regions of 4q13 and 9q31.

Conclusions: The results are inconclusive at this time. While FISH studies are encouraging, small sample size, incomplete molecular studies and IHC errors prevented decisive evidence of NR4A3 as a sensitive marker for AcCC.

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INTRODUCTION

Next generation sequencing has elucidated recurrent driver translocations in many tumors including salivary gland tumors [1, 2]. Detection and clinical testing for these driver translocations can improve diagnostic yield in challenging or ambiguous cases, characterize metastases of unknown origin, and offer the possibility for personalized medicine applications in patients who have failed conventional therapy or with high grade transformation. One such salivary tumor, Acinic Cell Carcinoma (AcCC) was recently found to confer a recurrent rearrangement [t(4;9)(q13;q31)]. This rearrangement allows for overexpression of Nuclear Receptor Subfamily 4 Group A Member 3 (NR4A3), enhancing cell proliferation and oncogenic behavior in AcCC [3, 4].

AcCC is typically a low-grade malignant salivary neoplasm accounting for 2-7% of all salivary malignancies [5, 6]. It occurs most commonly in the parotid glands, although other sites including the minor glands and the seromucous glands of the upper and lower airway are also sites of some tumors [7]. Like other salivary gland neoplasms, AcCC's have a slightly higher incidence in females than males (1.8:1). The age of presentation ranges from a mean of 40 years to 63.2 years [6, 7]. Although a typically low-grade tumor, 12% of patients with AcCC show metastasis to the cervical lymph nodes and lungs [8]. Rarely AcCC can undergo high grade transformation (de-differentiation) as reported by Thompson et al. (2015), and includes features such as high mitotic rate, perineural invasion, atypical mitoses and lymphovascular invasion. Median survival for patients with such high-grade tumors was 2.2 years [7]. Surgical excision is the treatment of choice with radiation for tumors presenting in advanced stages or incompletely

excised [8]. The prognosis for low-grade tumors is relatively good with a 5 year survival between 80-90% [8]. Serous acinar cell differentiation and zymogen granules are histologic hallmarks of this malignancy [6]. Four histologic variants are recognized: solid, microcystic, papillary cystic, and follicular. The mechanism driving these different histological variants are unknown [6]. Of the four, microcystic and solid are the most common [9, 2].

The majority of AcCC can be diagnosed using light microscopy and a limited histochemical and immunohistochemical (IHC) panel to distinguish AcCC from other salivary gland neoplasms [10]. For example, AcCC retains acinar cell-like differentiation with characteristic cytoplasmic periodic acid-Schiff (PAS)-positive zymogen granules resistant to diastase [11]. Special studies typically show that AcCC's are positive for DOG1 (also known as ANO1) and negative for S100, GATA3 and mammaglobin. Specifically DOG1 is routinely used as a marker of acinar cell differentiation with its characteristic luminal staining pattern [12]. However, recurrent cases presenting in atypical locations (i.e., outside the parotid gland), or those with oligometastasis may necessitate molecular characterization of the malignancy [7].

The majority of salivary gland neoplasms can be defined by their recurrent driver translocations with specific recurrent mutation hotspots in a minority of cases [1]. Molecular analysis can be used to distinguish several salivary gland neoplasms from AcCC, however specific driver balanced translocations or mutations for AcCC have only recently been described. For example: ETV6-NTRK3 gene fusion is found in 100% of secretory carcinomas (SC) [13, 14]. Adenoid cystic carcinoma (AdCC) commonly harbors the MYB-NFIB gene fusion [15]. CRTC1-

MAML2 fusions are frequently demonstrated in mucoepidermoid carcinomas (MEC) [16]. The first reported recurrent translocation in AcCC, HTN3-MSANTD3 fusion in a subset of AcCC's, was reported by Barasch et al., in 2017 [17]. However, Andreason et al., (2019), investigated a large cohort of 273 AcCC using fluorescence in situ hybridization (FISH) and real-time polymerase chain reaction (RT-PCR) for the recurrent HTN3-MSANTD3 fusion and found it in <5% of cases [11]. Haller et al., using genomic and epigenomic profiling, discovered a recurrent [t(4;9) (q13;q31)] rearrangement which enabled upregulation of NR4A3 as an initial oncogenic driver event in AcCC [3]. Until this discovery NR4A3 was a rearrangement exclusively found in extraskeletal myxoid chrondrosarcomas [18]. Additionally, Haller et al., reported that NR4A3 immunostaining was more reliable when compared with NR4A3 FISH [4]. Further research using next generation sequencing is needed to verify NR4A3 as an oncogenic driver and its association with the histologic subtypes and prognosis of AcCC [7, 11].

Next generation sequencing is a powerful tool to further validate NR4A3 as a possible driver mutation and/or a balanced translocation in AcCC [Figure 1]. Data obtained from this study, (8 cases from Walter Reed National Military Medical Center), may identify targetable mutations or pathways as was found in Secretory Carcinoma (SC), treated with Insulitn-like Growth Factor-1-Receptor (IGF1R) targeted linsitinib, a monoclonal drug therapeutic and Adenoid Cystic Carcinoma (AdCC) treated with a potent NOTCH1 inhibitor [3, 18]. Using formalin-fixed paraffin embedded (FFPE) materials, RNA and DNA sequencing was performed to understand transcriptional and genomic alterations. Additionally, fluorescence *in situ* hybridization (FISH) and IHC were performed to demonstrate genetic abnormalities and aberrant tumor protein expression respectively. These results may confirm the outcomes of Haller et al. [18], as well as

further describe the transcriptional alterations in these tumors. We also confirmed in a case with clear high grade transformation (HGT), that the NR4A3 was retained. By examining genomic and transcriptional changes in AcCC, our understanding of the transcriptional environment driving AcCC and possible druggable targets will be elucidated.

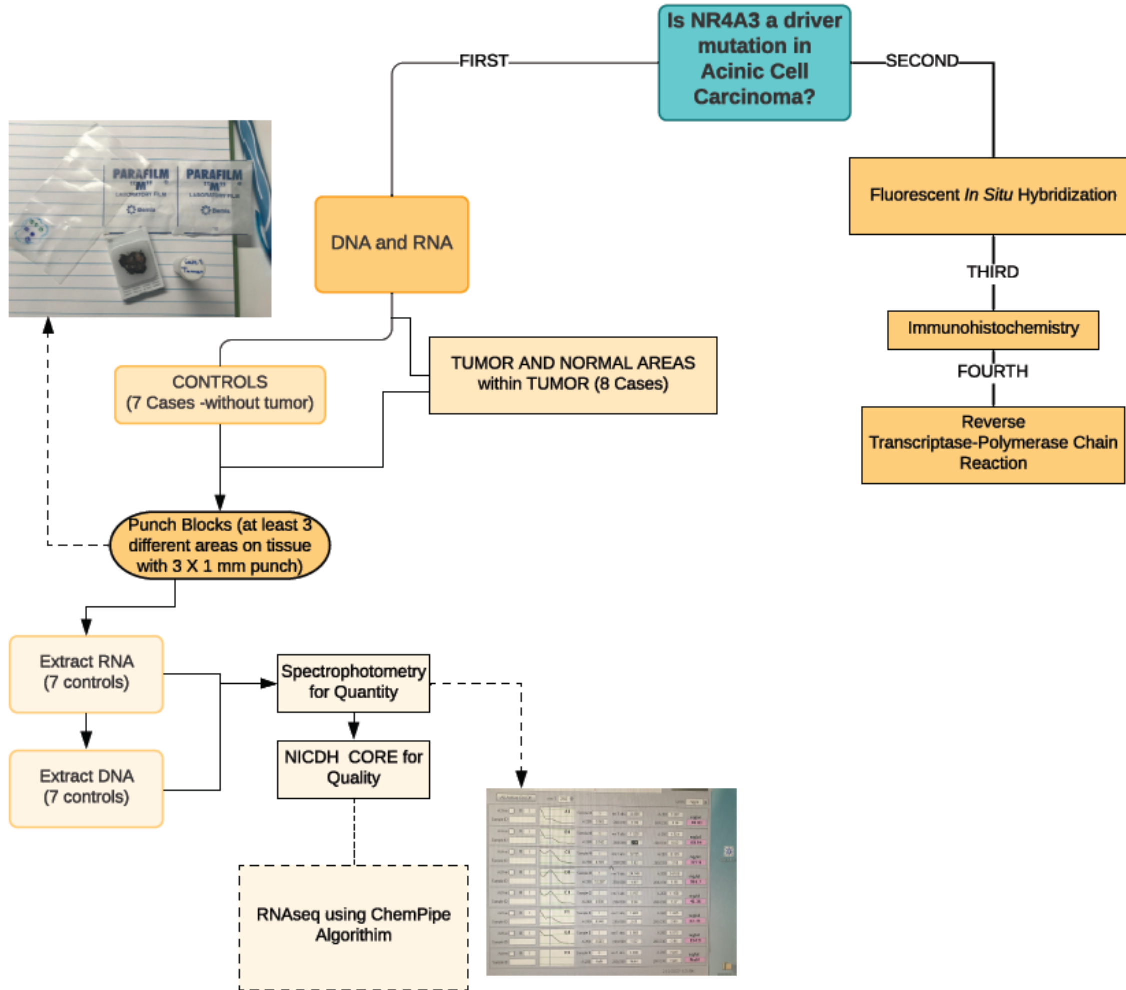

Figure 1 Overview of NR4A3 Elucidation

MATERIALS AND METHODS

This collaborative study was reviewed and funded by the Walter Reed National Military Medical Center (WRNMMC) #WRNMMC-2019-0240. In this study a total of 8 AcCC cases were collected from WRNMMC using CoPath Plus indexing system. Seven cases of normal parotid salivary gland were obtained from the National Institutes of Health (NIH).

Area Detection and RNA Extraction

Areas of tumor and normal tissue (normal adjacent parotid tissue where available) were identified by an Oral and Maxillofacial pathologist (Blake Warner (BW)) and an Oral and Maxillofacial Pathology resident, (Matthew Seedall (MS)), on hematoxylin and eosin (H&E) slides and marked with a marking pen. Parafilm (laboratory "M" biofilm) paper was then used to transfer marked areas (both tumor and benign) on slides to the formalin fixed paraffin embedded (FFPE) tissue blocks. The FFPE tissue blocks were then cored using 3 X 1 mm biopsy punches. RNA and DNA extraction were performed on the tissue biopsy samples (controls then tumors) using the Qiagen AllPrep DNA/RNA FFPE kit (Qiagen). RNA and DNA samples were assessed for quality and quantity using the Agilent Fragment Analyzer Capillar Electrophoresis system; DV200 scores were determined and scores >30% were used for sequencing. In order to detect cytogenetic translocations, RNAseq data will be analyzed using the ChimPipe algorithim. ChimPipe is a validated algorithim for detection of chimeric reads and enables identification of fusion genes.

Fluorescence In Situ Hybridization Method

Five of the eight tumor cases were chosen for FISH. The ZytoLight NR4A3 Dual Color Break Apart Probe kit (ZYTOVISION) was used for FISH studies. Briefly, 4 mm FFPE sections of tumor slides were used for FISH using 10 μL of NR4A3 ZytoLight FISH probe and counterstained with 4'6-Diamidino-2-Phenylindole (DAPI). The slides were incubated in the dark for 15 minutes and immediately examined using Olympus VS120 fluorescence microscope. In each case, 50 nuclei were counted. Samples with >20% abnormal signals (either split or single red signals) were considered positive. This same criteria for affirmative results was used by Haller et.al [4].

Immunohistochemical Studies

Immunohistochemical staining was performed using NR4A3 and ANO-1. NR4A3 was selected due to results published by Haller et.al. In their study 63 of 64 diagnosed AcCC cases showed moderate to strong nuclear expression using NR4A3 [4]. ANO-1 was chosen because it is a known immunohistochemical marker that consistently shows membranous (or luminal) positivity in AcCC [7]. The ImmPRESS Duet Double Staining Polymer Kit (Vector Laboratories) was used to perform immunohistochemistry following the manufacturer's recommended conditions. Slides used for NR4A3 primary antibody (1:100 dilution) were incubated overnight at 4ºC. The slides were washed in deionized water three times for ten minutes each. A 500 μL DAB EqV was created by mixing 250 μL of R1 and 250 μL of R2. The slides were subsequently incubated for ten minutes in ImmPRESS Duet Reagent followed by washing in 1X PBS buffer two times for five minutes each. The DAB EqV was then applied to the slides and allowed to incubate for five minutes. An ImmPACT Vector Red Substrate was then prepared (mixing 2.5 μL of Vector

Red diluent, 40 μL of Red R1 and 30 μL of Red R2) and added to each of the slides. The slides were then rinsed in 1X PBS buffer followed by distilled water (two times for five minutes each). The slides were counterstained with hematoxylin and tap water was used to rinse off the excess. ImmuMount was placed on each slide and coverslips added. Appropriate positive and negative controls were used.

RESULTS

Because of the retrospective nature of this study, no clinical history or follow up information was obtained for these cases. Of the fourteen cases, eight were selected (13 FFPE tissue blocks) due to amount of tumor available, metastasis, or histological features suggesting an aggressive nature. The histopathological diagnoses, age, and primary tumor location are summarized in Table 1. From the eight AcCC cases included five occurred in the parotid gland, one occurred in the left sphenoid sinus, another case was located in the right infra-auricular region and the last case was a metastasis from a primary located in the left parapharyngeal space to the left proximal humerus. Five of the patients were male and three were female. Their mean age was 56 years.

Table 1 Age, Location and Histopathological Diagnosis

CASE	AGE (y)//Sex	PRIMARY LOCATION	METASTASIS	HISTOPATHOLOGICAL DX
1	75/F	Parotid	-	ACC
2	26/M	Parotid (R)	-	ACC
3 M	69/F (PRIMARY) // 72 (METASTASIS)	Parapharyngeal space (L)	Proximal humerus (L)	ACC/ HG with M
4	80/M	Parotid	-	ACC
5	31/F	Infra-auricular (R)	-	ACC
6	46/M	Parotid	-	ACC
7 S	48/M	Sphenoid sinus (L)	-	ACC
8	52/M	Parotid (R)	-	ACC

M Metastasis; S Same location with different biopsy time; R Right; L Left; DX Diagnosis; ACC Acinic Cell Carcinoma; HG High Grade

Molecular Genetic Studies

RNA and DNA was extracted from seven controls and eight tumor cases. The DV200 (represents the percentage of RNA fragments >200 nucleotides and is an important quality index for next generation sequencing) is presented for the seven controls in Table 2 [19]. For the method of sequencing to be used (not completed at this time), at least 10 ng/ μL was necessary (using spectrophotometry). All seven controls were in range with the lowest concentration at 38.49 ng/ μL. All controls were sent to the National Institute of Bioinformation and Scientific Programming Core at NIH and came back with low quality RNA in two cases and low quality DNA in four cases.

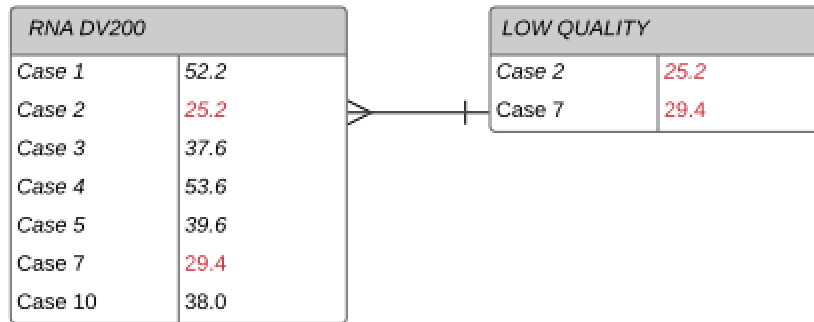

Table 2 RNA DV200 for Controls

Additionally, RNA and DNA was extracted from the eight tumor cases. When using the 3 X 1 mm punch on the tumor cases, areas of both normal and tumor were acquired. RNA and DNA (for the eight tumor cases) were assessed using the same method as used for the seven controls. All eight cases were within range with the lowest concentration for DNA (using spectrophotometry) at 11.8 ng/ μL and for RNA 12.86 ng/ μL. Both values came from normal areas of tissue. The contamination exceeded 2.0 for all samples of DNA (both tumor and normal). The RNA normal was all within range for contamination. RNA Tumor had one case with a contamination ratio of 2.05. All cases of RNA and DNA (both tumor and normal) were sent to the National Institute of Bioinformation and Scientific Programming Core. Results for quality have not yet been completed.

Histopathologic, Immunohistochemical, Fluorescent In Situ Hybridization

Six cases were composed of microcystic, tubular and solid growth patterns. These cases were composed predominantly of tumor cells with serous acinar features containing zymogen granules (no photomicrographs have been retained to create a figure). Often the tumor features were well-circumscribed (parotid cases). Perineural invasion, high mitotic rates and necrosis were not identified. With regards to the metastatic case, the overall features were microcystic but also dedifferentiated areas were identified.

Five cases based on adequacy of sample were chosen to perform immunohistochemical studies. Both ANO1 and NR4A3 staining were performed. ANO1 exhibited a positive membranous staining whereas NR4A3 showed positive nuclear features (no photomicrographs of the immunohistochemical stains have been retained to create a figure).

Of the five cases tested by FISH, four tested positive for the presence of NR4A3 genomic rearrangements (no photomicrographs have been retained to create a figure). FISH was performed using a dual-color break-apart probe. The positive results showed nuclei in interphase with one red and one green signal separated from each other. This corresponded with a positive translocation event at the NR4A3 gene locus.

DISCUSSION

The aim of this study was to confirm the main driver events in AcCC and to validate NR4A3 as a possible novel gene rearrangement and marker in eight cases of tumor (diagnosed as acinic cell carcinoma). Extraction of DNA and RNA yielded sufficient quantity in 5 cases of RNA and 3 cases of DNA (seven controls) to proceed to sequencing. The tumor samples were complete with regards to extraction of DNA and RNA but still need to be validated for quality at the core. It is critical to use samples of tumor with sufficient quantity and quality of RNA and DNA for sequencing to validate the main goal of the research. Haller et al. in a recent molecular study of NR4A3 noted that further studies were needed due to possible alternative genetic events such as the one done by Andreasen et al. on HTN3-MSANTD3 [10, 4].

Haller et al. demonstrated that 98% of 64 AcCC specimens displayed strong nuclear NR4A3 immunostaining. Their studies using immunohistochemistry for NR4A3 demonstrated a 100% specificity and a 98% sensitivity [4]. Of the five cases in which immunostaining was performed, strong nuclear staining (for NR4A3) was demonstrated but staining was also found in non-tumor areas. The same was true for ANO-1 which demonstrated a consistently positive membranous pattern as well as staining in non-tumor areas. This was due to failure at blocking endogenous peroxidase. While results were achieved, it would be important to perform the staining again for a more definitive outcome.

Although sensitivity and specificity were high for immunostaining in NR4A3, FISH sensitivity in the Haller et al. study was only 84% while looking at genomic rearrangements of the NR4A3

gene locus [4]. In the small sample size used (5 cases), 80% of the tumors were positive for the rearrangement. Split signals and single signals were observed in all cases with the exception of one case. The split is observed when individual red and green signal patterns are separated in cytological images. This split is seen because there is a break in the chromosomal regions of 4q13 and 9q31 as indicated by Haller et al. [4]. In order to further back-up FISH results, it will be important to validate molecular studies in this research.

CONCLUSION

The results of this research are inconclusive at this time. While the results of the FISH studies were encouraging, small sample size, incomplete molecular studies and immunohistochemical errors prevented decisive evidence of NR4A3 as a sensitive marker for AcCC.

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