The HTN3-MSANTD3 Fusion Gene Defines a Subset of Acinic Cell Carcinoma of the Salivary Gland

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Abstract: The spectrum of tumors arising in the salivary glands is wide and has recently been shown to harbor a network of tumor-specific fusion genes. Acinic cell carcinoma (AciCC) is one of the more frequently encountered types of salivary gland carcinoma, but it has remained a genetic orphan until recently when a fusion between the HTN3 and MSANTD3 genes was described in one case. Neither of these 2 genes is known to be implicated in any other malignancy. This study was undertaken to investigate whether the HTN3-MSANTD3 fusion is a recurrent genetic event in AciCC and whether it is a characteristic of one of its histological variants. Of the 273 AciCCs screened, 9 cases showed rearrangement of MSANTD3 by break-apart fluorescence in situ hybridization, 2 had 1 to 2 extra signals, and 1 had gain, giving a total of 4.4% with MSANTD3 aberrations. In 6 of 7 available cases with MSANTD3 rearrangement, the HTN3-MSANTD3 fusion transcript was demonstrated with real-time polymerase chain reaction. Histologically, all fusion-positive cases were predominantly composed of serous tumor cells growing in solid sheets, with serous tumor cells expressing DOG-1 and the intercalated duct-like cell component being CK7 positive and S-100 positive in 69 cases. All but one case arose in the parotid gland, and none of the patients experienced a recurrence during follow-up. In contrast, the case with MSANTD3 gain metastasized to the cervical lymph nodes and lungs. In conclusion, we find the HTN3-MSANTD3 gene fusion to be a recurrent event in AciCC with prominent serous differentiation and an indolent clinical course.

Key Words: carcinoma, acinic cell, salivary gland, salivary gland neoplasms, genes, HTN3-MSANTD3, gene fusion, genetics

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Salivary gland acinic cell carcinoma (AciCC) constitutes ~10% of primary salivary gland carcinomas and arises in female individuals in two-thirds of cases.1 Although typically a low-grade malignancy with a favorable long-term prognosis, the high-grade transformation is well described and is associated with an aggressive clinical course.2,3 With the exception of frequent DOG-1 (ANO1) expression, the immunohistochemical profile of AciCC is nonspecific, and the diagnosis is based on the characteristic acinar differentiation, which is aided by demonstration of cytoplasmic PAS-positive zymogen granules, which are resistant to diastase digestion.3,4 During the last decade, molecular studies have revealed numerous typespecific translocations in salivary gland tumors, which have become valuable diagnostic tools in equivocal cases and in cases of high-grade transformation, small biopsy specimens, so-called hybrid tumors, and even in fine-needle aspiration cytologies.5–7

In 2010, Skálová and colleagues separated a morphologically and genetically distinct subset of “zymogen granule-poor AciCC” from conventional AciCC, and they found this subset to be histologically and genetically identical to secretory carcinoma of the breast.5,8–12 However, only recently was “classic” AciCC shown to belong to the group of salivary gland carcinomas driven by fusion oncogenes.
with the demonstration of a gene fusion between the Histatin 3 (HTN3) and Myb/SANT-like DNA-binding domain containing 3 (MSANTD3) genes in an index case, and re-arrangement of MSANTD3 in 2/19 additional AciCCs by fluorescence in situ hybridization (FISH). Interestingly, rearrangement of the poorly characterized MSANTD3 gene was found to be unique for AciCC among all other types of salivary gland tumors. However, the HTN3-MSANTD3 fusion was demonstrated in the index case only, and the prevalence of this novel gene fusion in AciCC has been uncertain due to the relatively small sample size in this initial report. Moreover, MSANTD3 expression was not sensitive or specific for AciCC. Hence, the diagnostic, as well as prognostic, value of this gene fusion has remained unclarified.

In this study, we interrogated a large set of salivary gland AciCCs for the presence of MSANTD3 rearrangement and characterized cases with aberrations for expression of the HTN3-MSANTD3 fusion transcript. Moreover, we present the clinicopathologic data on the MSANTD3-rearranged AciCCs in order to ascertain the clinical course of this genetically distinct subset of AciCC.

MATERIALS AND METHODS

Patient Material

AciCC were retrieved from the surgical pathology archives from the authors’ institutions, including the material previously included by Barasch et al. Formalin-fixed paraffin-embedded (FFPE) blocks from 273 cases, including 5 cases with high-grade transformation, were retrieved and tissue microarrays constructed for the majority of cases after different protocols, either including large single cores or duplicate/triplicate 0.6 mm cores. Whole slides were evaluated for the remaining cases. Clinical information was collected from patient files. The study was approved by the Danish Data Protection Agency (J.no. REG-94-2014) and the Ethics Committee of the Capital Region of Denmark (J.no. H-6-2014-086 add. 58080).

Histochemistry and Immunohistochemistry

From MSANTD3-rearranged cases and 20 non-rearranged controls, whole sections were cut to 4 μm slides and stained with hematoxylin and eosin (H&E), periodic acid-Schiff with (PAS+D) and without (PAS-D) diastase, phosphotungstic acid hematoxylin (PTAH), and Perls’ prussian blue using standard protocols. Immunohistochemistry was performed as previously described using the antibodies listed in Table 1. Briefly, sections were mounted on coated slides, deparaffinized using EZ-prep, and the antigens retrieved with CCI in Tris buffer (pH 8.5) (all Ventana Medical Systems, Tucson, AZ). The ultraView DAB Detection kit (Ventana) was used for visualization, and slides were counterstained with Mayer’s hematoxylin. Positive controls as suggested on datasheets were used on each slide, and the expected reaction and subcellular site were confirmed. Positive and negative controls were used on all slides.

Fluorescence In Situ Hybridization

In order to screen for MSANTD3 rearrangement, the material was characterized using either a custom break-apart FISH probe for MSANTD3 generated from bacterial artificial chromosomes flanking MSANTD3, CTD-3186120 Cy5 (telomeric), and CTD-2363K7 Cy3 (centromeric) (BACPAC Resources Center, Children’s Hospital Oakland Research Institute) labeled with either Cy5 or Cy3 (GE Healthcare Life Sciences, Chicago, IL) or a commercially available break-apart probe (Empire Genomics, Buffalo, NY), which was hybridized using Vysis (Abbott Molecular, Des Plaines, IL) reagents and protocols. After hybridization, nuclei were counterstained with DAPI II (ZytoVision, Bremerhaven, Germany), and the images were captured using Ariol software (Applied Imaging, San Jose, CA). For each case, 60 to 100 nuclei were evaluated and rearrangement defined as ≥ 1 split signal in ≥ 10% of nuclei. The gain was defined as 2 of both signals in ≥ 10% of nuclei.

Ribonuclease Acid Sequencing

Total ribonuclease acid (RNA) was prepared for RNA sequencing in accordance with the manufacturer’s instructions (Illumina, San Diego, CA). RNA sequencing was performed using Illumina’s TruSeq Stranded Total RNA Library Prep Kit, and paired-end sequencing was performed to gain an output of 100 M reads according to the manufacturer’s instructions. FusionMap was used for the screening of fusion transcripts.

Real-time Polymerase Chain Reaction and Nucleotide Sequence Analysis

Total RNA was isolated from FFPE sections using Maxwell RSC FFPE kit (Promega, Madison, WI) according to the manufacturer’s instructions. RNA was converted to complementary deoxyribonucleic acid (cDNA) using the Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA) and performed according to the manufacturer’s instructions. RNA integrity was tested using primer pairs for the phosphoglycerate kinase gene (PGKI) in each case, as described previously. The cDNA was subjected to real-time polymerase chain reaction (RT-PCR) using the primers TGAGACTTCACTTCAGCTTCAC (NM_000200) and CAACGAAATTATAAAGCCTGCCA (NM_080655) spanning the fusion site between exon 1 and exon 2 of HTN3 and MSANTD3, respectively. PCR conditions were as follows: 94°C for 90 seconds followed by 40
cycles of 94°C for 45 seconds, 60°C for 1 minute, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. T3 and T7 tails were attached to the forward and reverse primers, respectively, which were used for later sequencing of the amplified products. The amplified fusion products were visualized using Qiaxcel (Qiagen GmbH, Hilden, Germany), and the 160 bp amplicons were subsequently sequenced using BigDye Terminator 3.1 Cycle Sequencing Kit, T3/T7-primers, and an ABI 3500 Dx DNA sequenator according to the manufacturer’s instructions (Thermo Fisher Scientific). Ten nonrearranged AciCCs were used as controls.

### Table 2. Demographics, Presentation, Treatment, and Outcome of Patients With MSANTD3 Aberrated Acinic Cell Carcinoma

<table>
<thead>
<tr>
<th>Case#</th>
<th>FISH</th>
<th>MSANTD3</th>
<th>Age (y), Sex</th>
<th>Site</th>
<th>Size (mm)</th>
<th>Presentation</th>
<th>Stage</th>
<th>Treatment</th>
<th>Outcome, follow-up (mo)</th>
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<tr>
<td>1</td>
<td>Break</td>
<td>HTN3-MSANTD3</td>
<td>59, M</td>
<td>Parotid</td>
<td>30×35×30</td>
<td>Parotid mass</td>
<td>T2N0M0</td>
<td>Surgery</td>
<td>DOC 98</td>
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<td>2</td>
<td>Break</td>
<td>HTN3-MSANTD3</td>
<td>52, F</td>
<td>Parotid</td>
<td>80×110</td>
<td>Parotid mass</td>
<td>T3N0M0</td>
<td>Surgery+RT</td>
<td>NED 269</td>
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<td>3</td>
<td>Break</td>
<td>HTN3-MSANTD3</td>
<td>51, F</td>
<td>Parotid</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>4</td>
<td>Break</td>
<td>HTN3-MSANTD3</td>
<td>75, F</td>
<td>Parotid</td>
<td>25</td>
<td>Parotid mass</td>
<td>T2cN0M0</td>
<td>Surgery+RT</td>
<td>NED 120</td>
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<tr>
<td>5</td>
<td>Break</td>
<td>HTN3-MSANTD3</td>
<td>65, F</td>
<td>Parotid, accessory</td>
<td>18</td>
<td>Buccal mass</td>
<td>T1cN0M0</td>
<td>Surgery</td>
<td>NED 36</td>
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<tr>
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<td>Break</td>
<td>HTN3-MSANTD3</td>
<td>28, F</td>
<td>Parotid</td>
<td>32</td>
<td>Incidental finding mass on MRI</td>
<td>T2cN0M0</td>
<td>Surgery</td>
<td>NED 24</td>
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<tr>
<td>7</td>
<td>Break</td>
<td>—*</td>
<td>59, M</td>
<td>Submandibular</td>
<td>25×20×25</td>
<td>Submandibular mass</td>
<td>T2N0M0</td>
<td>Surgery+RT</td>
<td>NED 58</td>
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<tr>
<td>8†</td>
<td>Break</td>
<td>HTN3-MSANTD3</td>
<td>76, F</td>
<td>Parotid</td>
<td>25</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Surgery</td>
</tr>
<tr>
<td>9†</td>
<td>Break</td>
<td>NA</td>
<td>49, F</td>
<td>Parotid</td>
<td>12</td>
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<td>NA</td>
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<td>Surgery</td>
</tr>
<tr>
<td>10†</td>
<td>Break</td>
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<td>30, M</td>
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<td>12 (recurrence)</td>
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<td>Surgery</td>
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</table>

*Absence of HTN3-MSANTD3 fusion transcript by RT-PCR and uninformative RNA sequencing.
†Cases previously reported by Barasch et al. Complex pattern, 1 to 2 extra green signals. Complex pattern, 1 to 2 extra red signals.
‡Fusion identified by RNA sequencing.
DOC indicates died of other causes; MRI, magnetic resonance imaging; NA, not available; NED, no evidence of disease; RT, radiotherapy.

![FIGURE 1. Patterns of MSANTD3 aberrations in acinic cell carcinoma of the salivary gland. A, FISH demonstrating separate green (arrows) and red (arrowheads) signals, consistent with fusion gene formation. The gain of one green signal (arrows) (B) and one red signal (arrowheads) (C) was present in a subset of cases.](image-url)
FIGURE 2. HTN3-MSANTD3 fusion transcripts in acinic cell carcinoma of the salivary gland. A, RT-PCR revealed a 160 bp fragment in cases 1 to 6 but not in case 7. RT-PCR did not yield an amplicon in any of the cases with complex MSANTD3 FISH patterns (not shown). All cases had PGK1 products, ensuring sufficient RNA quality (not shown). B, The breakpoints within the genomic location of the HTN3 gene on chromosome 4 and MSANTD3 gene on chromosome 9 with exons denoted by vertical bars schematically presented. An illustrative part of the fusion transcript demonstrating the fusion of exon 1 of HTN3 and exon 2 of MSANTD3, which was shown to be the same in all 6 cases with amplified PCR fragments by nucleotide sequencing. Note that the start codon (vertical arrow) lies within exon 2 of MSANTD3, causing for full-length MSANTD3 transcripts as marked by dark blue. Bp indicates base pair; M, marker; NC, negative control.

RESULTS

MSANTD3 Aberrations in AciCC

Among the 273 AcicCCs comprising the cohort, 9 cases (3.3%) showed split signals with the MSANTD3 probe in 17% to 48% percent of tumor cells, one case each showed 1 to 2 extra green and red signals, respectively, giving a total of 4% with MSANTD3 aberrations (Table 2 and Fig. 1). In addition to the index case from the study by Barasch et al13 (case 1, Table 2), RT-PCR identified the fusion between HTN3 exon 1 and MSANTD3 exon 2 in 6/7 rearranged cases with available tissue (lanes 1 to 6, Table 2, Fig. 2). Despite showing rearrangement of MSANTD3 by FISH and having sufficient RNA quality for RT-PCR, no fusion transcript was identified in case 7, and RNA did not meet quality requirements for RNA sequencing. One case that showed a gain of one MSANTD3 signal did not express a fusion transcript, which was also the case for 10 nonrearranged AcicCCs (Fig. 3).

Clinical and Histologic Findings in AciCC With MSANTD3 Aberrations

The clinical and pathologic findings of the cases are summarized in Table 2. Sex was known in 10/11 cases with 3 male patients and 7 female patients, with ages ranging from 28 to 76 years (mean: 54 y). All except one arose in the parotid gland and varied in size from 12 to 110 mm in largest dimension (mean: 33 mm). Stage information was available for 6/11 patients, and all presented with localized disease. Treatment information was available for 9/11 patients, and all underwent surgical resection of the primary site and 3 received adjuvant radiotherapy. Follow-up was available for 6/11 patients and ranged from 24 to 269 months (median: 78 mo). None of the patients experienced a recurrence during follow-up, although case 10 itself was a recurrent tumor for which no additional information was available on the primary tumor. The case with MSANTD3 gain arose in a 75-year-old woman who presented with cervical lymph node metastases and subsequently developed pulmonary metastases. She was lost to follow-up 3 months after pulmonary resection.

Only cores embedded in tissue microarrays were available in 4 cases, but the remaining cases ranged from well circumscribed (3/7) to locally invasive (4/7). All were lobulated lesions showing hallmark AciCC features with widespread to almost universally dominant neoplastic serous cells growing in solid sheets with pronounced cytoplasmic granulation (Figs. 3, 4A–D). Accordingly, the intercalated duct-like cell component was present in variable proportions ranging from minuscule to more conspicuous (Figs. 4C–F). Intercalated duct-like cells were variable in size and shape with eosinophilic to pale cytoplasm arranged in cysts, microcysts, and cribriform formations filled with a homogenous eosinophilic material (Figs. 4D–F). No areas of papillary or follicular growth or clear cell or oncocytic change were observed in any of the primary tumors, and none had lymphoid stroma or areas of high-grade transformation. Immunohistochemically, all available cases showed intense positivity for CK7 and S-100 in the intercalated duct-like component and intense expression of DOG-1 in the luminal membrane of serous tumor cells (Figs. 5A, B and Table 3). Alpha-amylase was expressed in the cytoplasmic granules of 6/10 cases (Fig. 5B, inset). Mammaglobin was consistently negative and Ki-67 index varied from 1% to 5% (mean: 2.2) (Table 3). This immunohistochemical profile was similar to what was found in 20 AciCCs without MSANTD3 aberrations (Table 3).

DISCUSSION

The landscape of tumor-specific gene fusions in salivary gland tumors has grown increasingly complex during the last decade and was recently expanded to include AciCC
In this case, was fused to a different exon of HTN3. Hence, we can only speculate whether the fusion partner, of this particular case due to insufficient RNA quality. sequencing failed to be informative about the fusion status harboring this fusion transcript by RT-PCR, and RNA one case arising outside the parotid gland (case 8) did not differentiate into this particular cell type. However, the intercalated duct-like cells were present in all cases, thereby suggesting an ability of the serous cell to differentiate into this particular cell type. However, the one case arising outside the parotid gland (case 8) did not harbor this fusion transcript by RT-PCR, and RNA sequencing failed to be informative about the fusion status of this particular case due to insufficient RNA quality. Hence, we can only speculate whether the fusion partner, in this case, was fused to a different exon of HTN3 or an entirely different gene, most likely a gene with a highly active promoter region.

In our expanded material of 273 cases, including the material from Barasch et al., we found MSANTD3 aberrations in a low proportion of AciCCs (4.4%), as compared with the 15% originally reported (Table 2). The histologic hallmark of AciCC is the serous cell component, which is present in variable proportions, together with the intercalated duct-like component growing in one or more different patterns, which include solid, microcystic, papillary-cystic, and follicular. Interestingly, with the exception of the case with isolated MSANTD3 gain, the AciCCs with MSANTD3 aberrations were all highly differentiated "classic" AciCCs dominated by serous tumor cells in a solid growth pattern. Moreover, none of the cases had significant lymphoid infiltrates, an otherwise common finding in AciCC and associated with a favorable outcome.

We found an identical immunohistochemical profile between our 12 cases and wild-type controls, distinctly different from that of secretory carcinoma, which was recently separated from AciCC (Table 3). Therefore, the archetypical features of AciCC with MSANTD3 rearrangement is solid growth, serous cell domination, and absence of lymphoid stroma. However, these characteristics are not unique to AciCC with MSANTD3 aberrations, and the clinical impact of identifying these patients is unlikely to be of significance, as the clinical course of the present material did not differ from the generally indolent nature of AciCC. Collectively, these findings do not merit the separation of MSANTD3-rearranged AciCC as a diagnostic entity, but rather as a genetic characteristic of a subset of classic AciCC.

Similarities between tumors of the salivary gland and breast are well known and include several entities in which identical fusion genes are found in histologically identical tumors of both sites, including adenoid cystic carcinoma and secretory carcinoma. Among the most intensely debated of these is breast AciCC, as it has a genetic profile similar to other triple-negative breast carcinomas with a high proportion of TP53 mutations, whereas the mutational burden in salivary AciCC is low. However, in contrast to most triple-negative
FIGURE 4. Histologic spectrum of salivary gland acinic cell carcinoma with MSANTD3 aberrations. A, Low-power magnification of a lobulated lesion composed of serous tumor cells in a solid growth pattern (H&E). B, High-power magnification of this cellular lesion showing these serous tumor cells with poorly defined borders, membrane-bound nuclei, and minimal intervening stroma (H&E). Minute cytoplasmic vacuoles are widespread, and hemosiderin granules are only rarely encountered (inset). C, Tumor cells showed occasional remnants of acinar arrangements and discrete cytoplasmic granulation readily discernable with PTAH (inset). Intercalated duct-like tumor cells line small luminae with eosinophilic secretion. Note the basally located nuclei in tumor cells growing along thin fibrovascular septae (H&E). D, Cytoplasmic granulation was present in all cases to a variable extent but was pronounced in most cases. The intercalated duct-like cell component had eosinophilic to pale cytoplasm, as exemplified by the central portion of this image (H&E). E, Low-power view showing larger cysts and cribriform growth mainly lined by intercalated duct-like cells with interspersed neoplastic serous cells (H&E). F, Most cases had areas of microcystic overgrowth with evenly distributed serous and intercalated duct-like tumor cells. Microcysts were filled with variable amounts of bubbly eosinophilic secretion (H&E).
breast carcinomas. AcicCC has a favorable prognosis similar to other salivary-type breast carcinomas found in the breast. The identification of a gene fusion involving the promoter region of the HTN3 gene, a gene not expressed in the breast, lends further support to AcicCC of the salivary gland and that of the breast as being distinctly different entities.

In conclusion, the HTN3-MSANTD3 fusion is a recurrent fusion event in the salivary gland AcicCC but is found in <5% of cases, in particular, those dominated by serous tumor cells in a solid growth pattern without lymphoid stroma. Although we report on relatively few cases of which one case with MSANTD3 gain metastasized, the clinical course of patients with MSANTD3 aberrations is not distinct from that of AcicCC in general. The molecular underpinnings for the vast majority of AcicCC remain unknown and await further investigation.

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REFERENCES


TABLE 3. Immunohistochemical Profile of Acinic Cell Carcinoma With and Without MSANTD3 Aberrations

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<thead>
<tr>
<th>Case #</th>
<th>Alpha-amylase</th>
<th>CK7</th>
<th>DOG-1</th>
<th>Ki-67</th>
<th>Mammaglobin</th>
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No material from case 11 was available for immunohistochemistry. NA indicates not available.


