A pilot study for presence of circulating tumour cells in adenoid cystic carcinoma


Abstract. Adenoid cystic carcinoma (ACC) is a rare salivary gland neoplasm with a poor long-term prognosis due to multiple recurrences and distant metastatic spread. Circulating tumour cells (CTCs) are tumour cells shed from a primary, recurrent, or metastatic cancer that are detectable in the blood or lymphatics. There is no literature to date confirming the presence of CTCs in ACC. The aim of this study was to determine whether CTCs are detectable in ACC. Blood samples were collected from eight patients with histologically confirmed ACC. The TNM stage of the tumour was recorded, as well as any prior treatment. CTCs were isolated by spiral microfluidics and detected by immunofluorescence staining. Three of the eight patients recruited (32.5%) had staining consistent with the presence of CTCs. Of these three patients with detectable CTCs, one had confirmed pulmonary metastasis, one had suspected pulmonary metastasis and was awaiting confirmation, and one had local recurrence confirmed on re-resection. One patient with known isolated pulmonary metastasis had previously undergone a lung metastasectomy and did not have CTCs detected. CTCs are detectable in ACC. In this small patient sample, CTCs were found to be present in those patients with recurrent local disease and known distant metastatic disease. CTCs in ACC should be investigated further for their potential use as an adjunct in staging, prognosis, and the detection of recurrence.

Introduction

Adenoid cystic carcinoma (ACC) is a rare malignant tumour of salivary gland origin with an incidence of 3–4.5 cases per 1,000,000 people. It accounts for 1% of all head and neck malignancies and 10–27.9% of all salivary gland tumours, and is one of the most common cancers of the major salivary glands. ACC occurs in all age groups, with a higher frequency in middle-aged patients (fifth and sixth decades of life), and has no identifiable risk factors. This carcinoma is characterized by perineural invasion and multiple recurrences. The prognosis is generally poor and therefore many clinicians consider ACC to be a ‘clinically high-grade’ neoplasm.

The treatment of ACC traditionally includes radical surgical resection...
whenever possible. This is typically followed by postoperative radiation therapy in the majority of cases. Regional lymph node metastasis is infrequent, reported to be found in 18.6% of all cases, but this may be a factor of infrequently performed neck dissections and lack of detailed assessment of retrieved lymph nodes. Haematogenous metastasis to lung, bone, and liver is common. Chemotherapy has been studied, but due to the indolent natural course of the disease, it has been difficult to observe the clinical response. Reported 5-year survival rates as high as 92% may give false optimism. Long-term overall survival rates at 10 and 20 years are 52–65% and 28–40%, respectively.

Circulating tumour cells (CTCs) are described as tumour cells detectable in the blood or lymphatics, and CTCs have been described in patients with head and neck cancers, among other cancer types. These cells are shed from primary, recurrent, or metastatic cancer. CTCs must undergo a complex transitional process known as epithelial to mesenchymal transition (EMT), which is the gateway to blood intravasation. CTCs can lose their epithelial antigens, gain migratory and invasive properties causing disruption of the basement membrane, and present with a mesenchymal-like phenotype. Once in the blood, platelets provide a microenvironment, forming a ‘platelet cloak’ that may shield CTCs from immune detection.

The presence of CTCs in peripheral blood is extremely rare, with detection in the order of 1 per 10⁸–10⁹ blood cells. The half-life of CTCs is as short as 2 hours in some solid tumours, as they are often destroyed by immunological processes, blood shear forces, or apoptosis. At the secondary site, CTCs have to change their new microenvironment in order for it to provide sufficient nutrients and oxygen, before they can redevelop as macrometastases. The reverse process of EMT, mesenchymal to epithelial transition (MET), is believed to play a role in allowing CTCs to ‘seed’ distant organs such as the lungs, liver, and bone, forming metastases. CTCs are therefore recognized as a key intermediate step in the development of tumour metastasis.

CTCs were first discovered in 1869, but it was not until 2005 that the clinical significance of the presence of these cells was proven. It was found that the presence of these cells was associated with a poor prognosis. The existence of CTCs in the bloodstream has been identified as a promising diagnostic, prognostic, and predictive biomarker (including for disease monitoring and the response to therapy) for a variety of human cancer types, including head and neck, lung, and breast cancers. In the staging of breast cancer, the presence of CTCs in cM0 disease is associated with an adverse prognosis for recurrence and survival. For those patients staged M1, a higher number of CTCs at the time of diagnosis has been strongly correlated to worse survival.

Investigations have been performed to determine the presence of CTCs in head and neck squamous cell carcinoma (HNSCC) patients, with CTCs being detected in 12.5–77% of these patients. This wide range is due to study heterogeneity, differing methods of detection, and the inclusion of all tumour stages. A decrease in CTCs within the first month following chemotherapy treatment for HNSCC has been found to be associated with longer progression-free survival and longer overall survival.

CTCs have been shown to have a relatively long tumour doubling time, on average 393 days, which may account for the poor long-term prognosis due to lung metastasis at >5 years after initial diagnosis. In fact, the onset of pulmonary metastasis has been hypothesized to be much earlier (227 months) than the initial visit. Given this early propensity for early metastasis, the presence of CTCs may heighten the search for occult distant disease. This may allow improved staging, or the detection of recurrence. It appears that no study to date has investigated the presence of CTCs in ACC. The purpose of this study was to investigate the possibility of detecting CTCs in ACC patients. A secondary aim was to determine the association between CTC detection rates and the clinical outcome in ACC patients, as this may offer an opportunity for the development of a precision medicine approach for these patients.

Materials and methods

Study design

This study was approved by the Ethics Committee of the Princess Alexandra Hospital, Brisbane, Queensland (HREC Number HREC/12/QPAH/381). A total of eight patients who had been diagnosed with ACC of the head and neck were recruited from the Oral and Maxillofacial Surgery Department, the Royal Brisbane and Women’s Hospital between October 2018 and May 2019. For all of these patients, the clinical stage of ACC was classified according to the American Joint Committee on Cancer/the Union for International Cancer Control (AJCC/UICC) tumour–node–metastasis (TNM) staging system (eighth edition of the AJCC Cancer Staging Manual). All participants gave written informed consent prior to sampling.

CTC enrichment by spiral microfluidics

CTCs were isolated from the blood samples of ACC patients using a spiral microfluidic-based technology, as described previously. Briefly, 10-ml whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) blood collection tubes and transported immediately to the laboratory for downstream processing. An initial red blood cell lysis (Astral Scientific Pty Ltd, Taren Point, NSW, Australia) was performed to minimize the cellular components passing through the spiral chip. The pellet was then resuspended in 10 ml of sheath buffer (1 x phosphate buffered saline (PBS), 2 mM EDTA, 0.5% bovine serum albumin (BSA)), before loading into a 10-ml syringe (Thermo Fisher Scientific, Rockford, IL, USA). Next, the samples were pumped through the spiral chip at a flow rate of 1.7 ml/min. Finally, both enriched CTC (CTC31) and depleted CTC/white blood cells (WBCs) (CTC42) were collected and fixed with 4% paraformaldehyde in PBS. After 10 minutes of fixation, both CTC18 and CTC25 were spun onto slides using a Cytospin centrifuge (Thermo Fisher Scientific) at 1200 rpm for 5 minutes. All of the glass slides were stored at −80 °C for subsequent analysis.

CTC characterization using immunofluorescence staining

Immunofluorescence staining of specific antigens for cancer cells and WBCs was performed for CTC characterization. Briefly, the glass slides were blocked with 2% BSA (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 1 hour at room temperature. The glass slides were then washed three times with PBS and incubated with human pan-cytokeratin (CK) eFluor 570-conjugated antibody (Thermo Fisher Scientific) and human CD45 aliphycocyanin (APC)-conjugated antibody (BD Biosciences, San Jose, CA, USA), as well as their respective isotype control antibodies. After 1 hour of incubation, the glass slides were washed three times with PBS and subsequently incubated with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes at room temperature before mounting with ProLong Antifade Reagents (Thermo Fisher Scientific). The fluorescence was determined using a Zeiss Axio Imager.
Z2 microscope (Zeiss, Oberkochen, Germany). CTCs were identified by the following criteria: CK-positive (green), CD45-negative (red), DAPI-positive (blue), a high nuclear-cytoplasmic ratio, and size larger than leukocytes. (Fig. 1)

Results

Eight patients with confirmed ACC had blood samples taken for analysis. The characteristics of these eight patients with histopathologically confirmed ACC are shown in Table 1. Four of the patients were female and four were male. The mean age at sample collection was 55.9 years (range 46–67 years). Six of the patients (75%) had an original tumour stage of T4. The most common primary site was the maxilla and/or palate (75%). Only one of the patients (12.5%) had evidence of regional metastasis on clinical imaging or final histopathology. Six of the patients (75%) had previously undergone surgical resection of the primary tumour, and in all instances this was followed by postoperative radiation therapy on recommendation of the hospital head and neck multidisciplinary team. Two of the patients (25%) had known metastatic disease, with biopsy-proven pulmonary metastatic deposits present. One of these patients had undergone a lung metastasectomy for the treatment of an isolated metastasis.

Three of eight patient samples (37.5%) stained positive for CK, negative for CD45, and positive for DAPI, consistent with the presence of CTCs. Of the three patients with detectable CTCs, one had confirmed pulmonary metastasis, one had suspected pulmonary metastasis and was awaiting confirmation, and one had local recurrence confirmed on re-resection. Five patients (62.5%) did not have CTCs detected with these staining methods. One patient with known isolated pulmonary metastasis had previously undergone a lung metastasectomy and did not have CTCs detected.

Discussion

ACC is a rare salivary gland neoplasm with a poor long-term prognosis due to multiple recurrences and distant metastatic spread1–3. In this pilot study, it was found that CTC detection was associated with the clinical disease status in ACC patients.

Based on the results of the staining, three of the eight patients (37.5%) were found to have CTCs. Patient 3, known to have untreated primary disease and two lung metastases on imaging, was found to have CTCs. These results could be expected given his clinical staging. Patient 6 was 5 years from his original surgical and postoperative radiation therapy. He has subsequently been diagnosed with mesothelioma. Interestingly, his latest chest imaging showed the presence of nodules not typical of mesothelioma, and he is set to have further investigations as this may represent metastatic ACC. Patient 7 had undergone two prior resections of the maxilla and nasal septum for the treatment of recurrent ACC, both followed by postoperative radiation therapy. She subsequently developed Notani grade 3 osteoradionecrosis of her entire remaining maxilla, necessitating a maxillectomy and composite free flap reconstruction. On histopathological analysis of the maxillectomy specimen, small foci of ACC were identified. The presence of CTCs in the setting of multiply locally recurrent ACC is in keeping with the aggressive nature of this particular tumour.

Of the five patients who did not have detectable CTCs, none had clinical evidence of local recurrence. Interestingly, patient 5 had undergone a lung metastasectomy for an isolated ACC metastasis only 1.5 months prior to sample collection. His sample did not demonstrate CTCs. This may demonstrate that metastasectomy is a reasonable option for isolated pulmonary metastasis, as has been argued previously4. The clinical value of CTCs for those undergoing curative intent pulmonary metastasectomy for colorectal cancer has been explored; it was.

Fig. 1. Detection of CTCs in adenoid cystic carcinoma patients. Representative fluorescent images of CTCs identified by the spiral microfluidic-based technology in combination with immunofluorescence staining on specific antigen for nuclear (DAPI, Blue), CTCs (CK, Green) and WBCs (CD45, Red). CTCs define as DAPI positive, CD45 negative and CK positive.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>TNM</th>
<th>Primary anatomical site</th>
<th>Previous local treatment</th>
<th>Local recurrence</th>
<th>Metastatic treatment</th>
<th>Active known disease</th>
<th>Presence of CTCs</th>
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<tr>
<td>1</td>
<td>67</td>
<td>F</td>
<td>pT4aN0M0</td>
<td>Nasal floor</td>
<td>Surgery + PORT Feb 2018</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>F</td>
<td>pT4aN1M0</td>
<td>Maxilla</td>
<td>Surgery + PORT Sept 2017</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>M</td>
<td>cT3N0M1</td>
<td>Sublingual gland</td>
<td>None</td>
<td>N/A</td>
<td>No</td>
<td>Yes (local and pulmonary)</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>M</td>
<td>pT4aN0M0</td>
<td>Palate</td>
<td>Surgery + PORT Dec 2018</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>No</td>
</tr>
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<td>5</td>
<td>48</td>
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<td>pT2N0M1</td>
<td>Palate (Lung nodule)</td>
<td>Surgery + PORT Dec 2013</td>
<td>No</td>
<td>Yes (lung metastectomy Dec 2018, prior to sample collection)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>M</td>
<td>pT4aN0M0</td>
<td>Maxilla (Lung nodule)</td>
<td>Surgery + PORT Sept 2014</td>
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<td>N/A</td>
<td>Yes (suspected pulmonary)</td>
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<tr>
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<td>pT4aN0M0</td>
<td>Palate</td>
<td>Surgery + PORT 2009, 2016, 2019</td>
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<td>N/A</td>
<td>Yes (local)</td>
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<tr>
<td>8</td>
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<td>F</td>
<td>cT4aN0M0</td>
<td>Palate into base of skull</td>
<td>None</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>No</td>
</tr>
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</table>

CTCs, circulating tumour cells; F, female; M, male; N/A, not applicable; PORT, postoperative radiation therapy.
*TNM staging as per the eighth edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual.\(^2\)
\(^b\) Presence of CTCs defined as staining CK-positive, CD45-negative, and DAPI-positive.

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**Ethical approval**

HREC/12/QPAH/381.

**Competing interests**

The authors have no conflicts of interest to declare.
Patient consent
Written patient consent was obtained prior to specimen collection.

References


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