Hydrogen Sulfide-Synthesizing Enzymes Are Altered in a Case of Oral Cavity Mucoepidermoid Carcinoma

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Hydrogen sulfide · Oral cavity · Mucoepidermoid carcinoma

Abstract
Mucoepidermoid carcinoma (MEC) is the most common malignant epithelial neoplasm of the salivary glands. MECs of the mouth floor are rare, with only a few cases reported. Here we report a MEC of the mouth floor in a 55-year-old woman. Since several studies have shown that hydrogen sulfide (H2S)-synthesizing enzymes are often increased in malignant tumors compared to benign counterpart tissues, we used western blotting to compare the protein levels of cystathionine-β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopuruvate sulfurtransferase (3-MST) in a mouth floor MEC to adjacent benign oral mucosa. We also used high-performance liquid chromatography to quantify possible differences in tissue sulfur fraction concentrations between the two biopsy types. Last, we used western blotting to examine nicotinamide phosphoribosyl transferase (Nampt), mitoNEET, and phospho-ser727-Stat3 levels in the biopsies. We found that all the proteins and phospho-ser727-Stat3 are increased in the MEC compared to benign mucosae. Interestingly, free H2S levels, acid-labile, and the sulfane sulfur factions were essentially the same between the MEC and benign tissue. Although
limited to a single and unusual tumor type, to our knowledge this is only the third time H$_2$S concentrations were directly quantified inside a human tumor. Last, our results replicate those of two previous studies where the H$_2$S-synthesizing enzymes are increased in a malignant tumor, while free H$_2$S is either not increased or only slightly increased, suggesting that malignant tumors rapidly metabolize H$_2$S as part of tumor maintenance and growth.

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Introduction

Mucoepidermoid carcinoma (MEC) is a malignant epithelial neoplasm of the salivary glands that arises from excretory duct pluripotent cells. This tumor type was first described by Volkmann in 1895, further analyzed by Massao and Berger in 1942, and described as a separate pathological entity by Stewart in 1945 [1]. MECs constitute approximately 35% of major and minor malignant salivary gland tumors and constitute approximately 40% of parotid, 7% of submandibular, and 3% of sublingual gland malignant tumors [2]. In the minor salivary glands, the palate and retromolar areas are common places for MECs, with only a few cases reported involving the mouth floor [1, 2]. MECs are common in the sixth decade, often presenting as slow-growing, painless lesions, with a male:female ratio of ~1.5:1 [1, 2]. MECs often carry a t(11;19)(q21;p13) translocation creating a MECT1-MAML2 fusion protein which activates the transcription of the Notch target gene HES1, contributing to cell growth and division [3]. Histologically, MECs are composed of cords, sheets, and clusters of mucinous, epidermal squamous, and poorly differentiated intermediate cells that have the ability to differentiate into either mucinous or epidermal cells [1, 2, 4] (Fig. 1). Low- and high-grade forms occur, with the low-grade form showing mucin-producing cells and a cystic architecture and the high-grade forms consisting of mainly epidermoid cells with increased pleomorphism, an infiltrative growth pattern, and an increased mitotic index [1, 2, 4]. Immunohistochemically, MECs are positive for CK7, CK14, mucicarmine, and antimitochondrial antibodies [2, 4, 5]. Here, we describe a MEC from the mouth floor where we measured tumor and adjacent benign oral mucosal free hydrogen sulfide (H$_2$S), and the acid-labile and sulfane sulfur fractions, and levels of H$_2$S-synthesizing enzymes.

Case Report

A 55-year-old woman presented with a history of hypertension, asthma, and a growing lesion on the right mouth floor. She denied pain or tongue numbness and stated that the lesion occasionally bled. She also had right neck swelling that was painful on palpation. The patient was consented for surgery and, with an Institutional Review Board approval, the biopsies were taken. At surgery, a 1.5-cm area was marked circumferentially around the tumor with Bovie electrocautery. Three 4-mm punch biopsies were obtained from the periphery of the margin and three 4-mm punch biopsies of the central MEC tumor core were taken. These were immediately placed in marked Eppendorf tubes and placed in a liquid N$_2$ bath. Less than 20 s passed between taking the punch biopsy and the biopsies being placed in liquid N$_2$. A right neck dissection was performed and obtained 31 lymph nodes.

We use the term “benign oral mucosa” to describe the benign tissue punch biopsies, as the half-life of H$_2$S within tissues is approximately 2 min, making a microdissection of the tissue not possible if H$_2$S tissue concentrations were to be properly analyzed [6]. The samples
were passed to the Pathology Department for further analysis. Upon histopathological analysis, a diagnosis of a high-grade MEC was rendered and the lesion was staged at Pathologic Stage pT2b, pN2b, Stage IVA. The right neck dissection revealed 5 lymph nodes positive for tumor (5/31). Representative H&E sections of the tumor are shown in Figure 1a and b. To further analyze the MEC, we performed western blotting on the MEC/benign mucosal tissue pair for CBS, CSE, 3-MST, nicotinamide phosphoribosyl transferase (Nampt), mitoNEET, and phospho-ser727-Stat3. The western blots were performed as previously described [7]. As shown in Figure 2a–c, the enzymes that synthesize H$_2$S (CBS, CSE, and 3-MST) were increased in the MEC compared to benign oral mucosa. Additionally, Nampt, mitoNEET, and phosphorylated ser727-Stat3 were all increased in the MEC compared to benign oral mucosa (Fig. 2d–f). Last, we measured bioavailable free H$_2$S, acid-labile, and bound (sulfane sulfur) levels, as previously reported [7]. As shown in Figure 3, all three portions of the sulfur pool were largely unchanged between the MEC and benign tissue samples.

**Discussion**

Here, we report a rare case of a mouth floor MEC. As there are few examples of this tumor type, we decided to analyze the H$_2$S-synthesizing enzymes CBS, CSE, and 3-MST, along with Nampt, mitoNEET, and phospho-ser727-Stat3, and directly measure the free H$_2$S, acid-labile, and bound (sulfane sulfur) cellular fractions. H$_2$S is a recently discovered gasotransmitter that promotes increased cancer cell growth and cell cycle progression, metastasis, invasion, angiogenesis, and chemotherapy resistance [6–8]. We found that the H$_2$S-synthesizing enzymes were increased in the MEC compared to benign oral mucosa (Fig. 2a–c). Although the activities of CBS, CSE, and 3-MST were not measured, the increased expression of these enzymes suggests that MECs increase H$_2$S synthesis as part of their pathobiology. Our finding that Nampt is increased in the MEC is not surprising, as Nampt is increased in many malignancies and functions, in part, as a regulator of CBS and CSE (Fig. 2d) [7, 9, 10]. Similarly, mitoNEET was increased in the MEC compared to benign tissue (Fig. 2e). MitoNEET suppresses apoptosis, autophagy, and lowers intramitochondrial iron concentrations, likely allowing tumor cells to tolerate higher reactive oxygen species, while avoiding ferroptosis [7, 11]. Lastly, we found that phospho-ser727-Stat3 was increased in the MEC compared to benign tissue (Fig. 2f). Stat3-ser727 phosphorylation increases CSE expression in breast cancer and may contribute to MEC tumor growth by increasing CSE protein levels [7].

We found that the sulfur fraction (free H$_2$S, acid-labile, and sulfane fractions) was nearly identical between the MEC and benign tissue. Only a small and statistically nonsignificant attenuation of the sulfane (protein-bound) sulfur fraction was identified (Fig. 3). This finding is interesting as CBS, CSE, and 3-MST proteins were increased in the MEC (Fig. 2a–c). Previously, we found that 15 cases of oral squamous cell carcinoma had elevated CBS, CSE, and 3-MST protein expression compared to benign oral mucosa, while the averaged free H$_2$S was significantly, but also only slightly, increased in these tumors [7]. Additionally, in a previous case report of an oral cavity adenoid cystic carcinoma free H$_2$S concentrations were significantly lower in this tumor compared to benign oral mucosa, while the acid-labile and sulfane fractions were not significantly changed [12]. Although the data here is limited to one rare malignancy, taken together these three studies lend support to the hypothesis that malignant cells produce increased H$_2$S and rapidly metabolize it to support tumor growth and maintenance. The acid-labile (iron bound, largely mitochondrial) sulfur fraction was very large in both the MEC and benign tissue. The reason for this is unknown, although possibly the high number of
mitochondria seen in MECs in ultrastructural studies and a field of cancerization effect may play a role [5]. The obvious limitation of this study is that only a single rare tumor type was examined. Our results suggest that dysregulated H₂S metabolism is part of MEC biology. Further studies are needed.

**Statement of Ethics**

The authors have no ethical conflicts to declare.

**Disclosure Statement**

The authors declare no conflict of interest.

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Fig. 1. Low-power (a) and high-power (b) images of the MEC tumor by H&E staining.

Fig. 2. Western blot analyses of benign oral mucosae and the oral MEC for CBS (a), CSE (b), 3-MST (c), Nampt (d), mitoNEET (e), and phospho-ser727-Stat3 (f). The protein control used for phospho-ser727-Stat3 was an antibody to whole Stat3 protein [7].
Fig. 3. Comparison of the cellular H\textsubscript{2}S pools of benign oral mucosae and the MEC case. Free H\textsubscript{2}S pool (blue), the acid-labile fraction ("iron-bound" fraction, red), and the bound (sulfane sulfur) pool (green).