Original Article
Increased nicotinamide phosphoribosyltransferase and cystathionine-beta-synthase in oral cavity squamous cell carcinomas

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Abstract: Background: Oral squamous cell carcinoma is a major cause of cancer-related deaths world-wide. Histologically it arises from the benign squamous epithelium lining the oral cavity and is conventionally divided into well, moderate, and poorly differentiated subtypes. Nicotinamide phosphoribosyltransferase catalyses the rate-limiting step of nicotinamide adenine dinucleotide synthesis and is highly expressed in many malignancies. Cystathionine-beta-synthase synthesizes hydrogen sulfide and shows increased expression in several malignancies. The expression of both enzymes and cellular hydrogen sulfide levels are known to cooperate to increase tumor survival and promote tumor dedifferentiation. Methods: We employed tissue microarray studies to analyze nicotinamide phosphoribosyltransferase and cystathionine-beta-synthase protein levels in oral squamous cell carcinoma. One-hundred and fifty-one different oral squamous cell carcinomas were analyzed for nicotinamide phosphoribosyltransferase protein levels and 233 oral squamous cell carcinomas were analyzed for cystathionine-beta-synthase protein levels. Results: The expression of both proteins is increased in oral squamous cell carcinoma and is also increased with increasing squamous cell carcinoma grade. Conclusions: Nicotinamide phosphoribosyltransferase and cystathionine-beta-synthase are both increased in oral squamous cell carcinoma and likely cooperate to promote oral squamous cell carcinoma growth and cancer progression. Additionally as both enzymes, particularly cystathionine-beta-synthase, increase with increasing squamous cell carcinoma grade, our data further suggests that higher expression of both enzymes promote tumor dedifferentiation.

Keywords: Nicotinamide phosphoribosyltransferase, cystathionine-beta-synthase, hydrogen sulfide, oral cavity, squamous cell carcinoma

Introduction

World-wide head and neck cancer is the sixth leading cause of cancer deaths, with 50% of afflicted individuals dying of the disease largely through post-therapy local recurrence, metastases, and diagnosis at advanced stages [1-3]. Approximately 90% of these tumors are squamous cell carcinomas (SCC) which arise from the stratified squamous epithelium lining the oral cavity [3]. Oral SCC arises from multiple, stepwise molecular alterations resulting in a progression from premalignant to invasive SCC [2, 3]. These changes include loss of heterozygosity at multiple genomic loci, epigenetic alter-ations, and gene mutations/amplifications, including pRb, p16, p53, epidermal growth factor receptor, Stat3, cyclin D1, AKT, and mTOR alterations [2, 4-10].

Recently the gasotransmitter hydrogen sulfide (H_2S) has been found to play a role in several human cancers, where it promotes cancer progression by accelerating cell cycle progression, ablating apoptotic responses, and promoting angiogenesis [11]. H_2S is synthesized by three different enzymes; cystathionine-beta-synthase (CBS), cystathionine gamma-lyase, and 3-mercaptopyruvate sulfurtransferase, and all three enzymes are increased in different malig-

nancies [11]. Presently the role of H_2S in oral SCC is unknown, although treatment with the H_2S donor NaHS accelerates oral SCC cell cycle progression in culture by activating the AKT kinase, suggesting that H_2S may promote this malignancy [12, 13]. Oral SCC also commonly shows Stat3 activation, an inducer of the enzyme nicotinamide phosphoribosyltransferase [Nampt, 14]. Nampt catalyses the rate-limiting step of nicotinamide adenine dinucleotide (NAD^+) synthesis and is increased in a number of human malignancies [15]. Nampt and H_2S form a positive feedback loop with H_2S inducing Nampt protein expression leading to cancer cell survival and dedifferentiation [16, 17]. Based on the data above we hypothesized that the Nampt and CBS proteins would be overexpressed in oral SCC. We chose to examine CBS expression over the other two H_2S-synthesizing enzymes, as it is increased in prostate, colon, ovarian, and breast cancers, and therefore might be over-expressed in oral SCC [14, 18-21]. Herein we used tissue microarray (TMA) technology to test this hypothesis.

Materials and methods

Upon Institutional Review Board approval by LSU Health Shreveport, tissue microarrays (TMAs), catalog numbers OR208 and OR2081, were purchased from US Biomax, Inc. (Rockville, MD, USA). Two of each of the TMAs were purchased, so that they could both be interrogated with anti-Nampt and anti-CBS antibodies. Together the TMAs contained 48 benign oral cavity squamous epithelial samples and 258 oral SCC samples; 214 SSC grade I, 30 SSC grade II, and 14 SCC grade III tumor samples. All tissue samples in the two TMAs were in 1.0 mm diameter.

Nampt and CBS immunohistochemistry (IHC)

The concentration of primary Nampt and CBS antibodies were optimized to normal kidney as control tissue. The staining of the TMAs was performed in the Tissue Core Histology Lab Facility at the Moffitt Cancer Center. The microarray slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per the manufacturer's protocol with proprietary reagents. Briefly, the slides were deparaffinized on the automated system with EZ Prep solution (#950-100; Ventana Medical Systems). The

heat-induced antigen retrieval method was used in Cell Conditioning 1 (#950-124; Ventana Medical Systems). Mouse monoclonal antibody to human Nampt (#ALX-804-717; Enzo life Sciences, Plymouth Meeting, PA, USA) was used at a 1:1000 concentration in Dako antibody diluent (#S0809; Dako, Carpenteria, CA, USA) and incubated for 60 min. The Abcam mouse monoclonal antibody to human CBS (ab54883, Cambridge, MA, USA) was used under the same conditions. The Ventana anti-mouse secondary antibodies were used for 16 min. The detection system used was the Ventana OmniMap kit. Slides were then dehydrated and cover-slipped as per standard laboratory protocol.

Evaluation of Nampt and CBS staining

Relative Nampt and CBS protein expression was determined as immunostain intensity scored on a 0-3 scale as follows: no staining as 0, light staining as 1, moderate staining as 2, and heavy staining as 3. The percentage of cells stained was measured, with no detectable staining as 0, 1-33% as 1, 34-66% as 2, and 67-100% as 3. The final IHC score was the product of the percentage of cells stained multiplied by the intensity score, allowing for a maximal score of 9 and a minimal score of 0. Nuclear and cytoplasmic Nampt and CBS staining were seen in all tissue samples examined, although at low levels in benign oral epithelium. We therefore measured and quantified Nampt and CBS staining in the nuclear and cytoplasmic compartments.
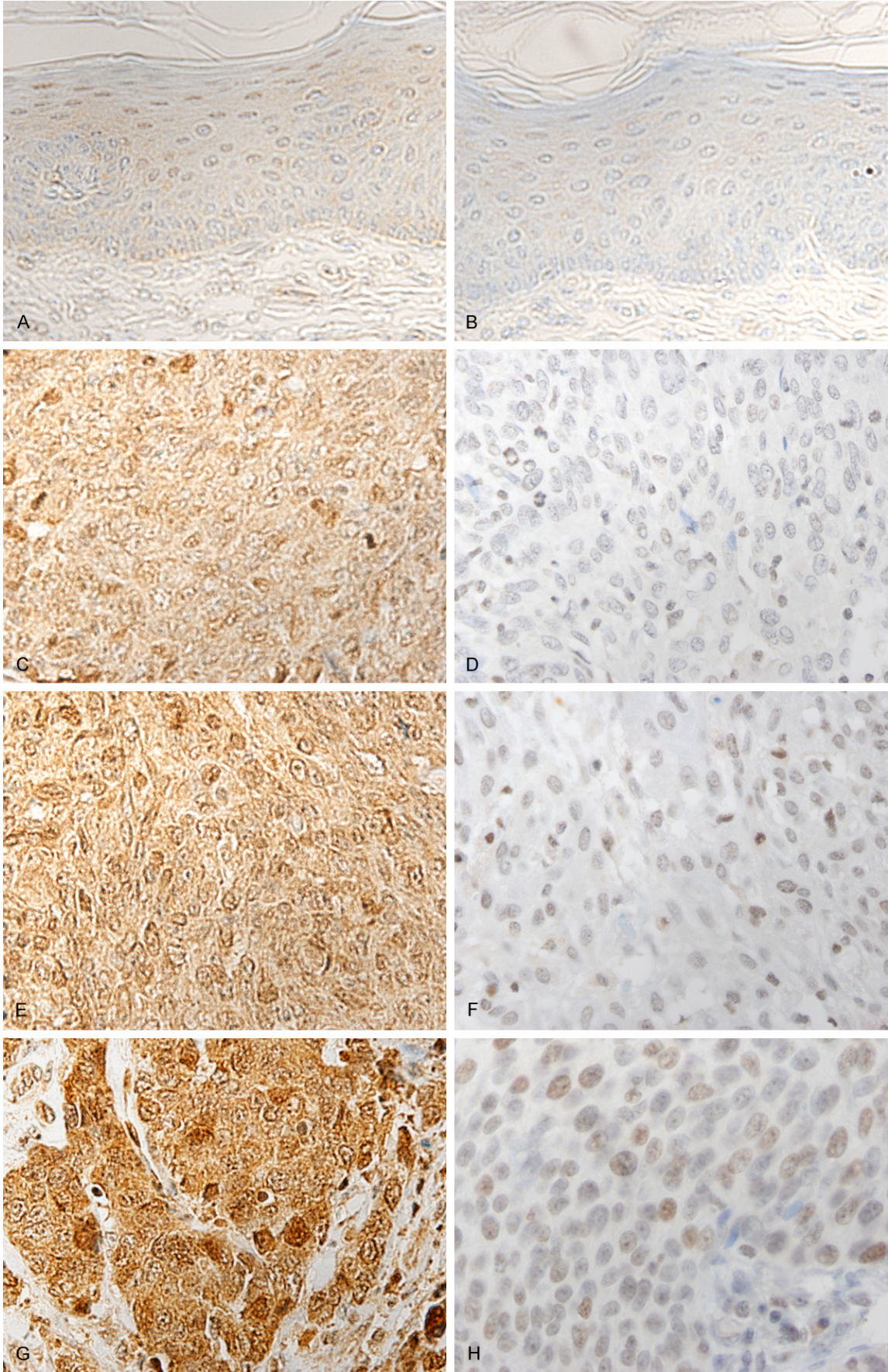
Statistical analysis

The standard error of the mean (SEM) IHC score was calculated by taking the mean of each data set, subtracting the mean from each number in the set and squaring the result, then calculating the mean of the squared differences, and taking the square root of this number to find the standard deviation. The standard deviation was then divided by the square root of the number of tissue samples in the sample set to find the SEM.

Results

Following IHC processing, we were left with 21 benign oral squamous epithelial samples and 111, 28, and 12 SCC grades I-III samples,

Figure 1. High-power views of Nampt and CBS IHC. Benign oral squamous epithelium Nampt (A) and CBD (B), grade I SCC Nampt (C) and grade I SCC CBS (D), grade II SCC Nampt (E) and grade II CBS (F), grade III SCC Nampt (G) and grade III SCC CBS (H).

Table 1. Representative high-power views of Nampt and CBS IHC staining on benign oral squamous epithelium and oral SSCs

Tissue Type	Sample Number		Average IHC Score		SEM	
Antibody Used	Nampt	CBS	Nampt	CBS	Nampt	CBS
Benign Oral Squamous Epithelium	21	37	2.87	0.56	0.51	0.13
SSC Grade I	111	192	4.69	1.21	0.99	0.10
SSC Grade II	28	29	5.75	3.52	0.45	0.49
SSC Grade III	12	12	7.20	5.75	0.42	0.94

IHC: Immunohistochemistry; SEM: standard error of the mean.

respectively for the TMAs probed with the anti-Nampt antibody. For the anti-CBS antibody TMAs we were left with 37 benign oral squamous epithelial samples and 192, 29, and 12 SCC grades I-III samples, respectively. Some cases were lost in IHC processing; hence we analyzed a fewer number of cases than were on the TMAs. Several tissues on the TMA were not analyzed due to their low numbers or irrelevance to this study. Examples of Nampt and CBS IHC of sample tissues are shown in **Figure 1**. The number of cases examined with each antibody, the quantified IHC results, and the SEM of each data set are given in **Table 1**.

Discussion

Here we show that Nampt and CBS are both increased in oral SCC compared to benign oral squamous epithelium and the expression of both proteins increases with SCC dedifferentiation (i.e., increasing tumor grade). Interestingly, Nampt expression was higher than CBS in all SCC tumor grades, and CBS expression was only moderately increased in grade I SCC compared to benign squamous epithelium and expressed at much greater levels in grades II and III SCC (**Table 1**).

Nampt expression is increased in several human malignancies including astrocytomas, well-differentiated thyroid, carcinomas, malignant lymphomas, colorectal carcinoma, ovarian serous adenocarcinoma, gastric cancer, prostate cancer, endometrial adenocarcinoma, melanoma, myeloma, leiomyosarcomas, and rhabdomyosarcomas [15, 22]. Nampt is more highly expressed in leiomyosarcomas than leiomyomas, with high-grade leiomyosarcomas showing higher Nampt IHC than low- and inter-mediate-grade leiomyosarcomas [22]. Similarly Nampt levels are higher in clinically aggressive rhabdomyosarcoma histologic subtypes compared to less clinically aggressive rhabdomyosarcomas subtypes [22]. This data, combined with our findings here, supports the hypothesis that increased Nampt levels correlate with increased tumor clinical aggressivity and dedifferentiation.

Similarly CBS is increased in prostate, colon, ovarian, and breast cancers and increased Nampt and H_2S levels cooperate to promote tumor survival [16-21]. It's therefore likely that the increased CBS seen here in oral SCC plays a role in promoting SCC cell growth. Additionally increased Nampt, CBS, and H_2S all promote similar events, including AKT activation and increased cell proliferation, glycolysis, and NAD^+ production [15-25]. Nampt inhibition also lowers CBS expression and cellular H_2S levels, while CBS inhibition in turn lowers Nampt expression and cellular H_2S levels [16, 17]. Although the molecular mechanisms by which Nampt, CBS, and H_2S regulate each other are presently poorly understood, the above data combined with this IHC study supports a role for CBS and Nampt in the promotion of oral SCC growth and progression. Lastly, this study raises the possibility that a CBS/H_2S inhibitor might have value in treating oral SCC. Further studies on the role of Nampt, CBS, and H_2S in oral SCC are currently underway.

Acknowledgements

Disclosure of conflict of interest

None.

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