Cystathione β -Synthase Is Increased in Thyroid Malignancies

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Abstract. *Background:* Cystathione β -synthase (CBS) catalyzes the conversion of homocysteine and cysteine to hydrogen sulfide (H_2S) and cystathione, via the trans-sulfuration pathway. CBS protein expression levels are increased in several different human malignancies, with increased protein expression correlating with parameters such as tumor stage, anaplasia, metastases, and chemotherapy resistance. *Materials and Methods:* This study employed tissue microarrays to examine CBS expression in benign thyroid tissue, thyroid oncocyomas, thyroid follicular adenomas, and in follicular, papillary, anaplastic, and medullary thyroid carcinomas. *Results:* CBS expression was increased in all thyroid carcinomas types compared to benign thyroid tissue, but not in thyroid follicular adenomas or oncocyomas. A similar pattern was observed for nicotinamide phosphoribosyltransferase (NAMPT) tissue microarray analysis comparing thyroid adenomas and follicular carcinomas. *Conclusion:* For the first time, we showed that an H_2S -synthesizing enzyme plays a role in thyroid malignancies. Additionally, our data suggest that CBS and NAMPT immunohistochemistry may be useful in differentiating follicular adenomas from follicular carcinomas.

Thyroid cancer constitutes approximately 1% of all human malignancies and is the most common neuroendocrine malignancy. Approximately 85% of thyroid cancers are the

well-differentiated papillary (PC) and follicular carcinoma (FC) subtypes, with the remaining less common subtypes being anaplastic thyroid (AC) and medullary thyroid carcinomas (MC) (1). Compared to thyroid malignancies, thyroid nodules/neoplasms are very common, with ultrasound studies indicating that they occur in one- to two-thirds of certain populations (1-3). Well-differentiated follicular adenomas (FAs) are the most common benign thyroid neoplasms and are five-times more common than FCs (4). Microscopically, FA and FC have very similar features and cytologically they cannot be distinguished. FAs and FCs are currently differentiated by the presence of capsular invasion, vascular invasion, extrathyroidal tumor extension, lymph node or systemic metastases, or any combination of these events, seen with FCs but not in FAs (1, 5).

At the molecular level, FAs and FCs exhibit a similar spectrum of molecular alterations (1, 5-13). FCs often carry paired box gene 8/peroxisome proliferator-activated receptors rearrangements, phosphatase and tensin homolog, and phosphatidylinositol 3-kinase carcinoma mutations, and Kirsten rat sarcoma viral oncogene homolog (*KRAS*, *HRAS*, and *NRAS*) point mutations (5-8). FAs can exhibit similar molecular alterations, usually at lower frequencies (9). Interestingly, functioning FAs often have activating thyroid-stimulating hormone mutations (10-12). In a recent study, Jung *et al.* identified 11 novel cancer-related genes mutations in FAs and FCs, (enhancer of Zeste 1 polycomb repressive complex 2 subunit, speckle type BTB/POZ protein, neurofibromin 1, transcription factor 12, insulin like growth factor 2 mRNA binding protein 3, lysine methyltransferase 2C, transcription complex subunit 1, breast cancer type 1 susceptibility protein interacting protein C-terminal helicase 1, lysine demethylase 5C, cohesin subunit SA-2, and mitogen-activated protein kinase kinase kinase 3) (13), indicating that a large number of different molecular alterations occur in thyroid follicular neoplasms. Presently, molecular analysis is rarely used to differentiate FAs from FCs (13).

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Table I. Relative cystathione β -synthase (CBS) and nicotinamide phosphoribosyltransferase (NAMPT) staining in the three tissue microarrays comparing benign thyroid and different thyroid neoplasms.

Tissue type	Immunohistochemical score							
	Number of samples		Average		SD		SE	
	NAMPT	CBS	NAMPT	CBS	NAMPT	CBS	NAMPT	CBS
Benign thyroid	12	10	0.75	0.40	0.45	0.50	0.13	0.22
PC	12	69	7.0	3.13	1.47	2.25	0.43	0.27
FC	12	29	7.75	2.14	1.55	1.75	0.45	0.32
FA	12	26	0.91	0.42	0.51	0.50	0.15	0.10
AC	12	6	7.25	3.5	2.01	1.22	0.58	0.50
MC	0	6	-	3.17	-	2.85	-	1.16
Thyroid oncocyoma	0	4	-	0.75	-	0.50	-	0.25

AC: Anaplastic thyroid carcinoma; FA: follicular adenoma; FC: follicular carcinoma; MC: medullary thyroid carcinoma; PC: papillary carcinoma.

Cystathione β -synthase (CBS) catalyzes the conversion of homocysteine and cysteine to hydrogen sulfide (H_2S) and cystathione, *via* the trans-sulfuration pathway (14). CBS is increased in several human malignancies, including colon, ovarian, bladder, breast, renal cell, and oral squamous cell carcinomas (15-20). Higher CBS level correlates with increased clinical aggressiveness in several different malignancies (15, 16, 18, 20). Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step of nicotinamide adenine dinucleotide synthesis (21). NAMPT expression is increased in several tumor types, including colorectal, serous ovarian, breast, gastric, prostate, oral squamous cell, and thyroid cancer, and melanoma, malignant lymphoma, myeloma, and astrocytomas (15, 16, 21-27). NAMPT expression is positively correlated with the clinical aggressiveness, stage, grade, metastatic potential, and resistance to chemotherapy in different tumor subtypes (15, 16, 21-27). CBS and NAMPT co-regulate each other, with CBS inhibition suppressing NAMPT protein levels and NAMPT inhibition suppressing H_2S synthesis (28). Since NAMPT and CBS co-regulate each other, and CBS protein levels are increased in several different cancer types, we hypothesized that CBS would be increased in thyroid cancer (14-28). Additionally, as both CBS and NAMPT expression in some tumors correlate with clinical aggressiveness and other tumor behavioral parameters, we further hypothesized that CBS and NAMPT expression would be higher in FC than in FAs.

Materials and Methods

Tissue microarray (TMA). Three TMAs TH641, TH802a, and TH8010a were purchased from US Biomax, Inc. (Rockville, MD, USA). TH641 was interrogated by antibody to NAMPT, while the other two TMAs were interrogated by antibody to CBS. The TH641 TMA contained 12 samples each of benign thyroid, PC, FC, AC, and FA. Together the TH802a and TH8010a MTAs contained 10

benign thyroid, 70 PCs, 29 FCs, six MCs, 26 FAs, four thyroid oncocyomas, and six AC samples. All tissue samples in the TMAs were 1.5 mm in 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 at 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 (Ventana Medical Systems). The heat-induced antigen retrieval method was used in Cell Conditioning 1 (Ventana Medical Systems). Mouse monoclonal antibody to human NAMPT (Enzo Life Sciences, Plymouth Meeting, PA, USA) and mouse monoclonal antibody to human CBS (Abcam, Cambridge, MA, USA) were used at a 1:1,000 concentration in Dako antibody diluent (Dako, Carpinteria, CA, USA) and incubated for 60 min. 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 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 to 3 scale as follows: no staining: 0, light staining: 1, moderate staining: 2, and heavy staining: 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 minimal score of 0 and a maximal score of 9. Nuclear and cytoplasmic NAMPT and CBS staining were seen in all tissue samples examined, although at low levels in benign thyroid and FAs. Therefore, NAMPT and CBS staining in the nuclear and cytoplasmic compartments were measured and quantified.

Statistical analysis of TMA results. The standard error of the mean (SEM) IHC score was calculated by using the standard deviation for the staining scores of each tumor type and dividing this number by the square root of the sample size (24, 25).

Figure 1. Representative nicotinamide phosphoribosyltransferase immunostaining of benign thyroid (A), thyroid follicular adenoma (B), thyroid papillary carcinoma (C), thyroid follicular carcinoma (D) and thyroid anaplastic thyroid carcinoma (E). Original magnification $\times 400$.

Results

Following IHC processing, all the samples in TH641 were stained with NAMPT. In the TH802a TMA, one PC sample was lost. The number of cases examined with each antibody, the quantified IHC results and the SEM of each data set are given in Table I. Examples of NAMPT and CBS IHC of sample tissues are shown in Figures 1 and 2. As previously noted, NAMPT was increased in thyroid PC and FC, indicating that the TMA analysis is replicable and reliable

(22). Additionally, for the first time NAMPT was found to be increased in thyroid AC and only marginally increased in thyroid FAs compared to its expression in benign thyroid tissue (Table I and Figure 1).

CBS was increased in thyroid FC, PC, MC, and AC compared to benign thyroid tissue. Like NAMPT, CBS was only minimally increased in thyroid FAs compared to benign thyroid. Last, CBS was mildly increased in thyroid oncocytomas compared to benign thyroid (Table I and Figure 2).

Figure 2. Representative cystathione β -synthase immunostaining of benign thyroid (A), thyroid follicular adenoma (B), thyroid papillary carcinoma (C), thyroid follicular carcinoma (D), thyroid medullary carcinoma (E), thyroid oncocytoma (F), and thyroid anaplastic carcinoma (G). Original magnification $\times 400$.

Discussion

Herein we showed for the first time that CBS, and hence H_2S synthesis, plays a role in thyroid cancer, with CBS protein levels increased in thyroid FC, PC, MC, and AC compared to benign thyroid tissue. Interestingly, CBS levels were also very different between thyroid FC and thyroid FAs, showing five-times greater levels of protein expression in the FCs than FAs. Since NAMPT was found to be induced in thyroid FC and PC, and CBS and NAMPT co-regulate each other, we also examined NAMPT expression in thyroid FC vs. FA (22, 23, 28). Similar to CBS expression, in these two tumor types, NAMPT expression was seven-times greater in FCs compared to FAs. Taken together, these results suggest that CBS and NAMPT immunohistochemistry may have utility in differentiating between these two histologically similar thyroid lesions. Further research into this hypothesis is needed. Lastly, we also show that NAMPT is induced in thyroid AC, while CBS is only marginally increased in thyroid oncocytomas compared to benign thyroid tissues.

CBS proteins levels are induced in colorectal, breast, urothelial, oral squamous cell, bladder, and renal cell carcinomas (15-20). Our finding that CBS is also in the major thyroid carcinoma subtypes supports the hypothesis that CBS induction is a common event in human malignancies. Furthermore, since increased NAMPT expression in thyroid malignancies is increased at higher stages and in tumors with increased metastatic potential, the same may be true for CBS expression (23). Studies that are more detailed are needed to address this possibility.

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