

Nicotinamide Phosphoribosyltransferase and SIRT3 Expression Are Increased in Well-differentiated Thyroid Carcinomas

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Abstract. Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step of nicotinamide adenine dinucleotide (NAD⁺) synthesis. NAMPT expression promotes angiogenesis, DNA synthesis, cell growth and survival, and mitochondrial biogenesis and function. Sirtuin-3 (SIRT3) is an NAD⁺-dependent deacetylase which functions in conjunction with mitochondrial NAMPT to promote cell survival following genotoxic stress. NAMPT expression is increased in several human malignancies, while SIRT3 levels are increased in some malignancies and suppressed in others. Based on this, we hypothesized that NAMPT and SIRT3 expression might be increased in well-differentiated thyroid carcinomas (TCs), follicular carcinomas (FC) and papillary thyroid carcinomas (PTC). Immunohistochemical analysis for NAMPT and SIRT3 staining was performed on these tumors using tissue microarrays. NAMPT and SIRT3 expression was low in benign thyroid tissues, moderately increased in FC, and more highly expressed in PTC. Specifically we observed both NAMPT and SIRT3 to be highly expressed in well-differentiated TCs. The data suggest that mitochondrial alterations play a role in the development and maintenance of well-differentiated TC. Since an effective pharmacological NAMPT inhibitor is currently in clinical use, further studies of NAMPT overexpression in well-differentiated TCs may be useful in selecting patients for NAMPT inhibitor therapy, particularly for metastatic well-differentiated thyroid carcinomas refractory to other treatments.

Roughly 80-90% of thyroid carcinomas (TCs) are either papillary (PTC) or follicular thyroid carcinomas (FC), together termed well-differentiated TC variants (1). Although thyroid nodules are common, well-differentiated TCs are uncommon, making up fewer than 1% of all human malignancies. Typically these types of cancers are rare in the young population and their incidence increases with age. Most patients are diagnosed when they are 45 to 50 years old (2). PTC makes up approximately 80% of all TCs and is histologically characterized by nuclear elongation with crowding, overlap, irregular nuclear contours with nuclear grooves, intranuclear cytoplasmic pseudoinclusions, multiple micronucleoli, and peripheral chromatin clearing causing hypochromatic nuclei. FC makes up about 5% of all TCs. Its diagnosis requires differentiation from an adenoma by the identification of tumor extension through the tumor capsule or extracapsular vascular invasion. FC lacks the nuclear features of PTC and cellular histology plays little role in its diagnosis. Clinically, PTC is multicentric in 20-80% of patients and most often metastasizes to regional lymph nodes, while FC is less often multicentric and tends to have blood-borne metastases to the lungs and bone (2-4).

Several molecular alterations have been identified in well-differentiated TCs. PTCs exhibit high phosphorylation of Signal transducer and activator of transcription-3 (Stat3), mutation of v-raf murine sarcoma viral oncogene homolog B1 (BRAF)V600E, high expression of MET or MNNG HOS Transforming gene (C-MET), neurotrophic tyrosine kinase receptor type 1 (NTRK1), mutations of Rat Sarcoma (RAS), rearrangements/fusions of rearranged during transfection/papillary thyroid cancer (RET/PTC). FCs often have Paired box gene 8/ peroxisome proliferator-activated receptors (PAX8/PPAR) rearrangements and Phosphatase and tensin homolog (PTEN) and Phosphatidylinositide 3-kinases carcinoma (PI3KCA) mutations, and both TC types express

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increased Hypoxia-inducible factor-1 (HIF-1) (3, 5-8). Many of these molecular changes promote angiogenesis, cell-cycle entry, and the expression of cancer-associated gene products (3-11). Interestingly, Stat3 and HIF-1 expression promotes nicotinamide phosphoribosyltransferase (NAMPT) expression - an enzyme that catalyzes the rate-limiting step of the nicotinamide adenine dinucleotide (NAD⁺) synthesis salvage pathway (12, 13). NAMPT expression promotes cell growth and survival, DNA synthesis, mitochondrial biogenesis, and angiogenesis (14-20). NAMPT expression is increased in ovarian, gastric, colorectal, esophageal, and prostatic carcinomas, and in malignant astrocytomas (21-29). SIRT3 often functions as a tumor suppressor, although its expression is increased in metastatic breast cancer and human oral cancers (30). Interestingly, some studies indicate that NAMPT and SIRT3 function as a unit promoting cell survival following genotoxic stress and in coordinating tumor cell energy metabolism (20, 36).

Based on the above data, we hypothesized that NAMPT and SIRT3 expression would be increased in well-differentiated TCs. Here we demonstrated that NAMPT and SIRT3 expression is very low in benign thyroid tissues and is significantly increased in FC and PTC, with PTC expressing the highest NAMPT and SIRT3 levels.

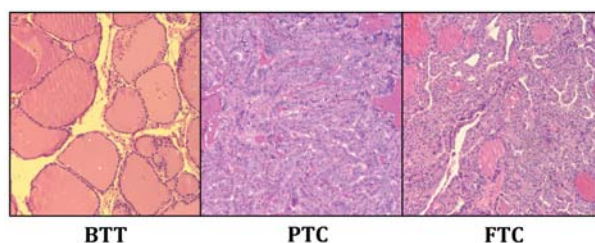
Materials and Methods

Human thyroid tissue microarrays (TMAs) were purchased from US Biomax, Inc. (Rockville, MD, USA), catalog numbers TH481 and TH801. The TH481 TMA consists of 48 thyroid tissue samples, 1.5 mm in diameter, including eight benign thyroid tissues (BTTs), 16 FC samples, and 24 PTC samples. The TH801 TMA consists of 80 thyroid tissue samples, 1.5 mm in diameter, including 40 BTTs, nine FC samples, 27 PTC samples, two medullary thyroid carcinoma samples, and one lymphoma, sarcomatoid, and squamous cell carcinomas each. Examples of hematoxylin and eosin (H&E)-stained benign thyroid tissues and FC and PTC were obtained from an H&E-stained TH801 US Biomax TMA.

NAMPT and SIRT3 Immunohistochemistry (IHC). The concentration of primary NAMPT and SIRT3 antibodies was optimized to normal kidney as control tissue. The staining of the TMA 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, slides were deparaffinized on the automated system with EZ Prep solution (#950-100; Ventana Medical Systems, Tucson, AZ). The heat-induced antigen retrieval method was used in Cell Conditioning 1 (#950-124, Ventana Medical Systems). The mouse monoclonal antibody to human NAMPT (#ALX-804-717; Plymouth Meeting, PA, USA) and the rabbit monoclonal antibody to human SIRT3 (#AP6242a; Wuxi Aptec, San Diego, CA, USA) were used at a 1:1000 concentration in Dako antibody diluent (#S0809; Dako, Carpinteria, CA, USA) and incubated for 60 min. The Ventana anti-mouse or rabbit secondary antibodies were used for 16 min. The detection system

Table I. Relative Nicotinamide phosphoribosyltransferase (NAMPT) and NAD-dependent deacetylase sirtuin-3 (SIRT3) staining in the two tissue microarrays comparing Benign Thyroid Tumor (BTT), Follicular Thyroid Carcinoma (FTC) and Papillary Thyroid Carcinoma (PTC).

Thyroid tissue type	Immunostain	Sample number	Staining	SEM
Benign thyroid tumor	NAMPT	48	1.29	0.01
	SIRT3	8	1.50	0.53
Follicular thyroid carcinoma	NAMPT	22	4.86	0.09
	SIRT3	16	4.00	0.11
Papillary thyroid carcinoma	NAMPT	49	8.19	0.05
	SIRT3	22	5.91	0.09



Medullary thyroid cancer was not included, due to the small sample size. The H&E image, of BTT, FTC and PTC were taken at magnification ×100.

used was the Ventana OmniMap kit. Slides were then dehydrated and coverslipped as per normal laboratory protocol.

Evaluation of NAMPT and SIRT3 staining. Relative NAMPT and SIRT3 protein immunohistochemical expression was determined as immunostain intensity scored on a 0-3 scale, with 3 being maximal. Immunostain intensity was scored with no staining being 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 percent of cells stained multiplied by the intensity score, allowing for a maximal score of 9 and a minimal score of 0. Nuclear and cytoplasmic staining was seen in all tissue samples examined, although at low levels in benign thyroid tissue. SIRT3 cytoplasmic staining was present but it was low in all tissues. An example of cytoplasmic expression of SIRT3 is shown as an inset in Figure 1, B(iv). We therefore measured and quantified NAMPT and SIRT3 staining in the nuclear and cytoplasmic compartments.

Statistical analysis. The standard error of the mean (SEM) 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. SEM calculations could not be performed on the medullary thyroid carcinomas, because there were only two samples for this category. Pearson's correlation coefficient for NAMPT and SIRT3 expression was calculated according to reference 37.

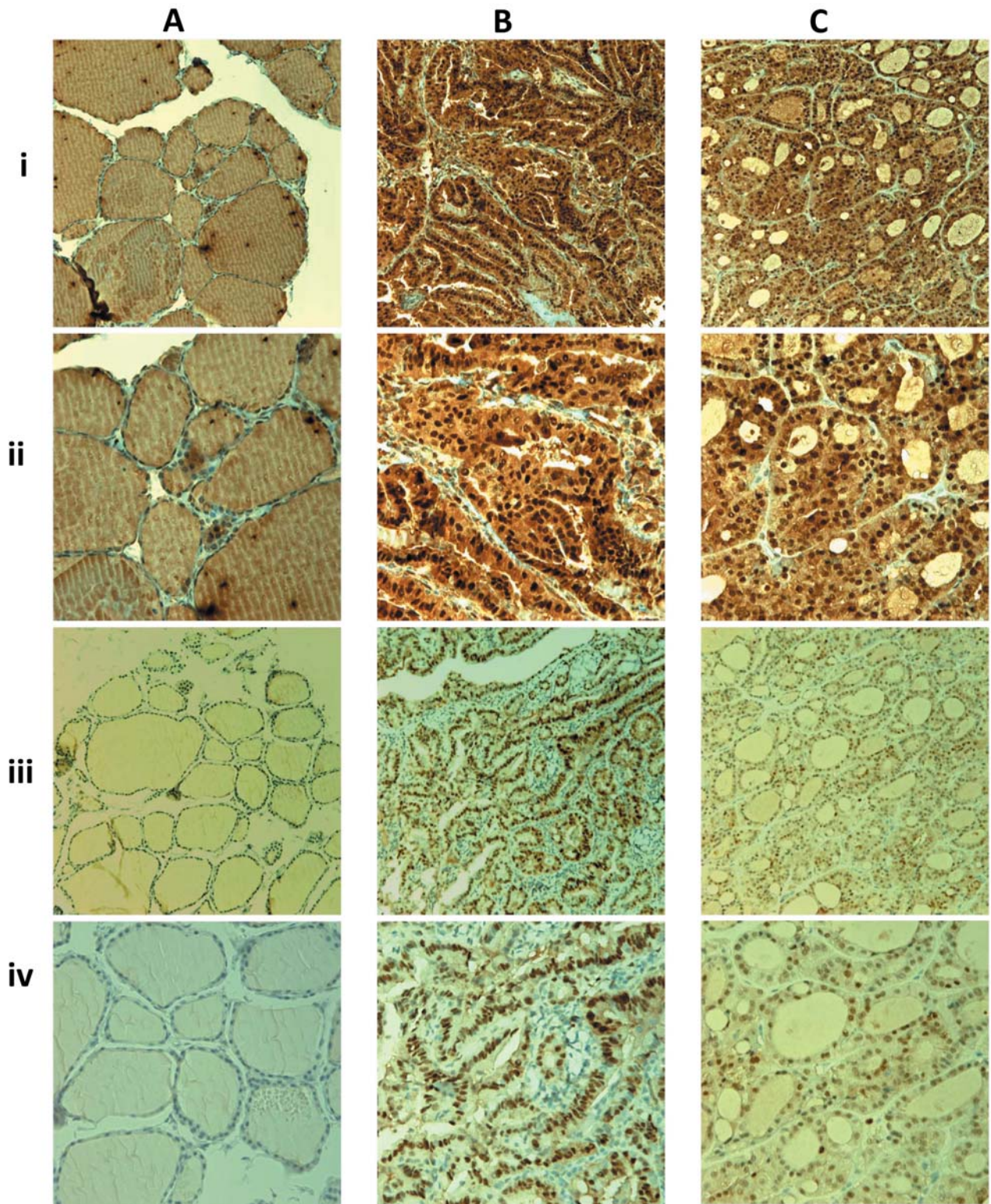


Figure 1. Representative immunostaining of benign thyroid tissue (A), papillary thyroid carcinoma (B) and follicular thyroid carcinoma (C) for Nicotinamide phosphoribosyltransferase (NAMPT) (i and ii) and NAD-dependent deacetylase sirtuin-3 (SIRT3) (iii and iv). i, iii: Low-power view, $\times 100$; ii, iv: high-power view ($\times 400$). The inset in B(iv) shows low cytoplasmic staining for SIRT3. Due to the low number of samples, medullary, squamous, sarcomatoid tumors, and the thyroid lymphoma are not shown. E is a close-up insert showing low cytoplasmic staining for SIRT3 immunostaining in PTC.

Results

Following the elimination of tissue samples lost during IHC processing, we were left with 48 BTT samples, 49 PTC cases, 22 FC cases, and two medullary thyroid carcinoma cases for NAMPT staining, and 8 BTT samples, 16 FC cases, and 22 PTC for SIRT3 staining. The lymphoma, sarcomatoid, and squamous cell carcinoma samples were not analyzed, as there was only one case for each of these malignancies. The number of each tissue type examined was lower than that on the TMAs due to some samples being lost. One TH481 TMA was subjected to NAMPT or SIRT3 IHC, and one TH801 TMA was subjected to NAMPT IHC. One fewer TMA was performed for SIRT3 IHC as clear differences were obtained with one TMA carrying a relatively large number of samples. Representation of the IHC results for NAMPT and SIRT3 are depicted in Figure 1.

The number of cases examined and immunohistochemical scores for each TC type is given in Table I. The typical H&E histology of each thyroid tissue type is also shown at the bottom of Table I. Pearson's Correlation Coefficient for NAMPT and SIRT3 expression was 0.89, demonstrating a strongly-positive correlation for the co expression of NAMPT and SIRT3 in well-differentiated TCs. These data do not demonstrate that the two proteins co-localize.

Discussion

Although both PTC and FC are well-differentiated TCs, their histology, clinical behavior, molecular characteristics, and epidemiology are significantly different (1-8). PTC exhibits increased Stat3 expression and both well-differentiated TC types exhibits increased HIF-1 expression (3, 7, 8). Stat3 and HIF-1 activity induce NAMPT, the enzyme that catalyzes the rate-limiting step of NAD⁺ synthesis (13-15). NAMPT expression promotes cell growth and survival, DNA synthesis, mitochondrial function, and angiogenesis and it is increased in a variety of human malignancies (15-29). Interestingly, mitochondrial NAMPT provides protection against cell death following genotoxic stress, by maintaining intact mitochondrial NAD⁺ levels, even when nuclear and cytoplasmic NAD⁺ levels are depleted, an event requiring SIRT3 expression (20). SIRT3 appears to function both as a tumor suppressor and a tumor promoter, depending on the tumor type (30).

The subcellular location of SIRT3 is controversial, with some research showing it to be exclusively mitochondrial, while others claim it is both nuclear and mitochondrial (31-33). Low mitochondrial SIRT3 correlates with increased glycolysis, common in malignancies, while its increased mitochondrial expression promotes cell survival following genotoxic stress (20, 33-35). Finely *et al.* (34) recently found that nuclear, but not mitochondrial SIRT3, is rapidly degraded with oxidative and UV-induced cellular stress, resulting in the expression of

stress-related and mitochondrial genes, suppressing events such as apoptosis. Thus it is possible that SIRT3-related tumorigenic events could involve subcellular compartmental-specific increases or decreases in SIRT3 expression and activity. In well-differentiated TCs, we found high nuclear and relatively low cytoplasmic SIRT3 (Figure 1Biv), indicating that at least for this tumor type, increased nuclear SIRT3 may play a role in maintaining these malignancies.

We hypothesized that well-differentiated TCs would have increased NAMPT and SIRT3 expression. Here we showed by TMA IHC that both NAMPT and SIRT3 expression are significantly increased in well-differentiated TCs (Figure 1 and Table I). Possibly NAMPT levels are also increased in medullary thyroid carcinomas, however, our sample size here was too small for a definitive conclusion to be drawn. Pearson's correlation coefficient for NAMPT and SIRT3 expression was found to be 0.89, indicating that these proteins are co-expressed in the same well-differentiated TCs. Further studies to assess co-localization of NAMPT and SIRT3 within the tumor cells are currently underway.

Our data indicate that NAMPT and SIRT3 are increased in TCs, and may play a role in altering mitochondrial NAD⁺ metabolism and in the development and maintenance of PTC and FC. Support for this view comes from previous studies showing overexpression of mitochondrial proteins and mitochondrial mutations in TC (38-40). Lastly, the specific NAMPT inhibitor FK866 has shown promising results in treating human cancer (41, 42). Since NAMPT is over-expressed in PTC and FC, FK866 may have value as an adjuvant therapeutic modality for PTC and FC refractory to other treatments. Further studies to explore this hypothesis are warranted.

Conflicts of Interest

The Authors report no conflicts of interest.

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