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ORIGINAL PAPER

GABA Receptor Expression in Benign and Malignant Thyroid Tumors

Stephen S. Roberts · Maria Cecilia Mendonça-Torres · Kirk Jensen · Gary L. Francis · Vasyl Vasko

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Abstract Neurotransmitter systems have recently been shown to be involved in multiple malignancies including breast, colon and prostate cancers. The role of neurotransmitters and neurotrophic factors has not yet been examined in thyroid cancer. To determine the possible involvement of neurotransmitter systems in thyroid carcinogenesis we characterized the patterns of gamma-aminobutyric acid (GABA) receptor expression in normal thyroid and thyroid tumors. We examined the expression patterns of the GABAergic system in 70 human thyroid tumor samples (13 follicular adenomas, 14 follicular carcinomas, 43 papillary carcinomas) and adjacent normal thyroid by immunohistochemistry. GABAergic system mRNA expression in thyroid cancer cell lines derived from primary (FTC133) and metastatic tumors (FTC236 and FTC238) was examined by real time PCR. Overall, GABA receptor expression is increased in tumors compared to normal thyroid tissue. Expression of GABAA receptor β2 was detected in the vasculature of normal thyroid and thyroid tumors but not in thyroid cancer cells. GABAA α2 was detected in metastatic-derived but not in primary-tumor derived cell lines. Expression levels of GABAB R2 and GABA receptor associated protein (GABARAP) are increased in adenomas and thyroid cancer suggesting their role in early stages of thyroid tumorigenesis. This study represents the first demonstration of GABA receptor expression in human thyroid tissue and suggests that the GABAergic system is involved in thyroid carcinogenesis.

S. S. Roberts (✉) · M. C. Mendonça-Torres · K. Jensen · G. L. Francis · V. Vasko
Department of Pediatrics,
Uniformed Services University of the Health Sciences,
4301 Jones Bridge Road,
Bethesda, MD 20814, USA
e-mail: sroberts@usuhs.mil

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Abbreviations

GABA	Gamma-aminobutyric Acid
GABARAP	GABA receptor associated protein
PCR	Polymerase chain reaction
FC	Follicular carcinoma
PC	Papillary carcinoma

Introduction

Thyroid cancer is the most common endocrine malignancy, with over 35,000 new cases diagnosed annually in the United States [1–4], and the incidence appears to be rising [1, 5]. While most patients have an excellent prognosis, a subset that develops distant metastases has aggressive disease and a poor prognosis [2, 6]. There remains a significant need for understanding of molecular mechanisms involved in thyroid carcinogenesis in order to develop effective therapy.

Recent evidence suggests that neoneurogenesis (ingrowth of nerve cells into a tumor and the actions of neurotrophic factors on tumor cells and the tumor microenvironment) plays a role in carcinogenesis in general, and in the initiation of metastasis development in particular [7, 8]. Neurotransmitter systems to include catecholamines (epinephrines and norepinephrines), neurokinins (Substance P), opioid peptides and their receptors (DOR, MOR) as well as GABA and its receptors have been shown to regulate cancer cell migration and the development of metastases [9].

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter of the mammalian nervous system. GABA receptors consist of three main receptor subtypes, $GABA_A$, $GABA_B$, and $GABA_C$, with the $GABA_A$ receptor being the most abundant [10].

Although primarily found in the central nervous system, GABA receptors are also widely expressed in both the peripheral nervous system and non-neural tissues [11, 12]. GABA appears to play a role in the homeostasis of the normal thyroid [13]. The thyroid gland has been shown to both actively take up and synthesize GABA [14, 15]. Research in rodent models suggests a complex interaction between thyroid hormone and GABA, with each regulating secretion or uptake of the other. Thyroid hormones appear to inhibit uptake of GABA in central nervous system neurons [16, 17], while GABA inhibits thyrotropin (TSH)-induced release of thyroid hormone from thyroid follicular cells [18].

A growing body of evidence also links the GABAergic system to human malignancy. GABA also appears to be involved in the regulation of cell migration and metastasis. Increased GABA content and GABA synthetic activity have been reported in breast, colon, gastric, ovarian and breast carcinomas [19–24].

Gene expression profiling of thyroid cancer revealed differential patterns of GABA receptor expression including $GABA_A$ $\alpha 2$ and $GABA_A$ $\beta 2$ receptor subunits [25]. These data suggested the possible involvement of the GABAergic system in thyroid carcinogenesis; however, no studies directly measuring GABA receptors in thyroid tissue have been published. In addition, we also chose to examine the expression of the $GABA_B$ receptor, and GABARAP, the main protein responsible for intracellular GABA receptor trafficking.

Here we report the expression patterns of this subset of GABAergic system genes in human thyroid tumors.

Materials and Methods

Patients and Tissues

Seventy tissue specimens were obtained from an archival tissue bank that is maintained under approval of Walter Reed Army Medical Center. The institutional review board has approved the protocol for this study and informed consents were obtained. Specimens were fixed in 10% formalin and embedded in paraffin. Histological sections of 5 μm thickness were stained with hematoxylin and eosin. Thyroid lesions were classified according to WHO criteria. There were 13 follicular adenomas, 14 follicular carcinomas (FC), 43 papillary carcinomas (PC) including ten cases of follicular variant of papillary carcinomas. Normal thyroid tissue adjacent to the tumor was available in 26 cases and was utilized as control tissue.

Thyroid Cancer Cell Lines

Human thyroid cancer cell lines derived from primary thyroid cancer (FTC 133), lymph node metastases (FTC 236) and distant metastases (FTC 238) were propagated in RPMI medium 1,640 supplemented with 5% FCS.

Immunohistochemistry

Sections were dewaxed, soaked in alcohol and incubated in 3% hydrogen peroxide for 15 min to inactivate endogeneous peroxidase activity after microwave treatment in antigen unmasking solution (Vector Lab, Burlingame CA). Sections were subsequently incubated overnight at 4°C with specific individual GABA system antibodies. The anti-GABARAP (sc 9190), $GABA_A$ $\alpha 2$ (sc 7350), $GABA_A$ $\beta 2$ (sc 7363), $GABA_B$ R2 (sc 22322) antibodies were from Santa Cruz Biotechnology, Inc. Immunostaining was performed with the Vectastain Universal Quik kit (Vector Lab, Burlingame CA) according to the manufacturer's instructions. Negative controls were performed by omission of primary antibodies.

The level of staining was quantified by two independent observer and staining intensity was scored as 0—no staining, 1—low staining and 2—focal intense staining and 3—diffuse intense staining.

Real Time Reverse-Transcriptase Polymerase Chain Reaction

RNA was extracted from thyroid cancer cell lines using the Trizol method (MRC Research, Inc) according to the manufacturer's instructions. RNA quality was verified by spectrophotometry prior to use.

The expression levels of $GABA_A$ $\alpha 2$, $\beta 2$, and $GABA_B$ R2 receptors and GABARAP were measured in each cell line. Matching primer and probe sets were designed using Primer Express software (Applied Biosystems, Foster City, CA). Full sequences for all primers and probes are shown in Table 1. One-step Quantitative PCR was performed in triplicate using 100 ng of total RNA per reaction. These were performed on an Applied Biosystems 7500 Real-time PCR system at 50°C for 38 min for the reverse-transcription, followed by 95°C for 15 s and 60°C for 1 min per cycle for a total of 40 cycles. Ribosomal 18S RNA was used as an endogenous control. Results were analyzed and relative fold-changes between transcript levels were calculated using the formula $2^{-}\Delta\Delta^{CT}$ (Comparative C_T method) per the manufacturer's instructions.

Table 1 List of genes and sequences of primers and probes used for quantitative RT-PCR

Gene name	GenBank accession number	Forward primer	Reverse primer	Probe
$GABA_A$ α2	NM_000807	AACACCGAATTCTGCTTGCC	GGTCCCACACCAAGAAAACAA	TCAGAGCGGCGGTGATGAAGACAA
$GABA_A$ β2	NM_021911	TGTCGCTGGTTAAAGAGACGG	AATGTTCATCCCCACAGCCA	TAGACTCCTGAAAGGCTATGACATTCGTCTGAGA
$GABA_B$ R2	NM_005458	CTGGTATTCGTGCCGAAGCT	GTGACCGAGGTGGACGTTTT	CGCAGAACAGGCGATTCCAGTTCA
GABARAP	NM_007228	AGGCTCCCAAAGCTCGGATA	AATTCGCTTCCGGATCAAGA	AAATACCTGGTGCCTTCTGATCTCACAGTTGG
18s rRNA	X03205	CGGCTACCACATCCAAGGAA	GGGCCTCGAAAGAGTCCTGT	CAGCAGGCGCGCAAATTACCCA

Sequences for the primers and probes are shown in 5′ to 3′ orientation. All probes contain a FAM fluorescent reporter on the 5′ end and an Iowa Black™ quencher on the 3′ end

Statistical Analysis

Statistical calculations were performed using SPSS 13.00 (SPSS, Inc, Chicago, IL). The significance of the differences between the means of variables was determined by Student's t-test. A probability value (P) of less than 0·05 was considered statistically significant. Pearson Correlation test was used to evaluate association between GABAergic system expression and pathological characteristics.

Results

$GABA_A$ Receptor Expression in Thyroid Tissue

The levels of $GABA_A$ β2 and $GABA_A$ α2 receptor subunit expression were assessed in thyroid tissue samples by immunohistochemistry.

Immunostaining with anti- $GABA_A$ β2 was restricted to vasculature in normal thyroid tissue. The highest level of staining was detected in the arteriolar compartment of microvasculature, especially in pericytes. The patterns of staining with anti-$GABA_A$ β2 in thyroid follicular adenomas and follicular cancer were similar to those observed in normal thyroid tissue and expression of $GABA_A$ β2 was restricted to the tumor vasculature. In papillary cancer, we observed vascular staining with anti- $GABA_A$ β2 antibodies; however, in five of 43 examined PTCs, $GABA_A$Rβ2 expression was detected not only in vessels but also in neoplastic epithelial cells. PTCs positive with $GABA_A$ β2 demonstrated solid architectural patterns of growth. See Fig. 1.

The expression of $GABA_A$ α2 was not detected in normal thyroid tissue samples. Most of the examined tumors showed no significant staining with anti- $GABA_A$ α2 receptor antibodies. Only two of 57 thyroid cancers (both PTCs) demonstrated significant cytoplasmic staining (Fig. 1).

$GABA_B$ R2 Expression in Thyroid Tissue

No significant staining for $GABA_B$ R2 was found in normal thyroid tissue. In contrast, increased intensity of staining with anti-$GABA_B$ R2 anti-bodies was found in a majority of thyroid tumors. High levels of $GABA_B$ R2 expression was detected in 4/13 FA and in 41/57 thyroid carcinomas (Fig. 1). The differences between expression of $GABA_B$ R2 in normal thyroid and thyroid tumors was statistically significant (p=0.001). The level of staining between benign and malignant thyroid tumors showed a trend towards statistical significance (p=0.07). There were no significant differences between follicular cancer and papillary cancers.

GABARAP Expression in Thyroid Tissue

No evidence of GABARAP expression was detected in 18 of 26 normal thyroid tissue samples. In the remaining eight cases, focal staining was observed in the cytoplasm of epithelial cells. The highest levels of GABARAP expression were found in epithelial cells located in areas of lymphocytic infiltration.

The level of GABARAP expression was significantly increased in thyroid adenomas and thyroid cancer compared to normal thyroid tissue (p=0.011 and p=0.001 respectively). The level of staining with anti-GABARAP antibodies was not significantly different between benign and malignant thyroid tumors as well as between follicular cancers and papillary cancers.

GABA Receptor Expression and Pathological Characteristics

No statistically significant association was seen between the level of GABA receptor expression, tumor size, extension of invasion, or presence of metastases.

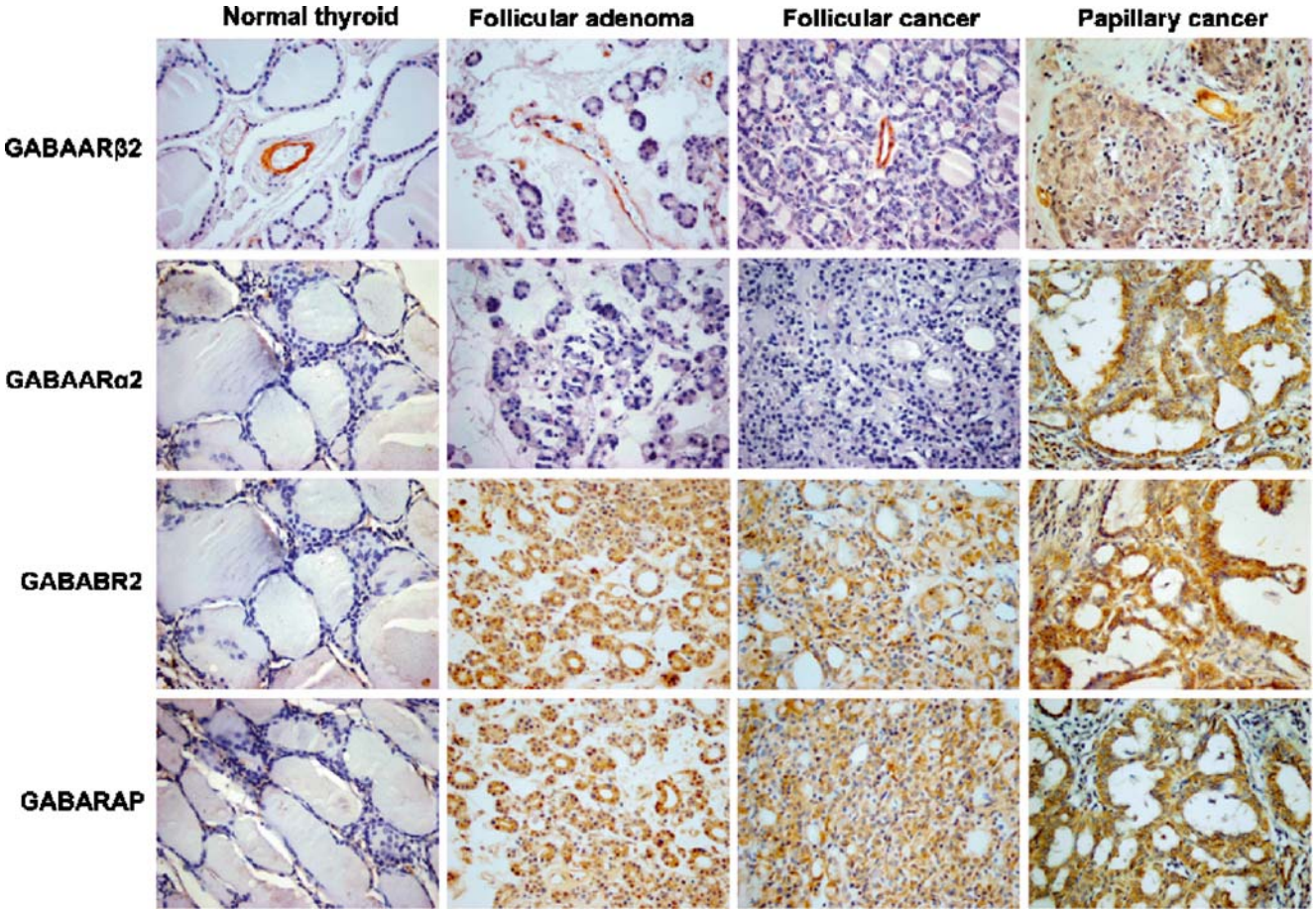

Fig. 1 $GABA_A$ β2, $GABA_A$ α2, $GABA_B$ R2 and GABARAP immunostaining in human thyroid tissue. $GABA_A$ β2 staining is detected in vasculature. $GABA_A$ α2 expression in papillary thyroid carcinoma. GABARAP and $GABA_A$ α2 are over expressed in follicular adenomas, follicular and papillary carcinomas

In Vitro GABA Receptor Expression

Real time PCR results were consistent with immunohistochemical data from human thyroid tumor samples. Specifically, mRNA coding for $GABA_A$ β2 was not detected in any of examined thyroid cancer cell lines. $GABA_A$ α2 mRNA expression was not seen in thyroid cancer cell lines derived from primary tumor (FTC133) but was detected in cell lines derived from metastatic tumors (FTC236 and FTC238). The mRNA for $GABA_B$ R2 and GABARAP was detected in all three thyroid cancer cell lines. The mRNA level of $GABA_B$ R2 was dramatically decreased in FTC236 and FTC238 (metastatic-derived) cells compared to FTC 133 (primary tumor-derived) cells by 1,000- and 490-fold respectively. The mRNA level of GABARAP was decreased in FTC236 and FTC238 (metastatic-derived) cells compared to FTC 133 (primary tumor-derived) cells by 1.4 and 1.3-fold respectively.

Discussion

GABA and its receptors have been widely studied in the central nervous system since GABA was first discovered more than 50 years ago. Only more recently, however, has it become clear that GABA is a ubiquitous signaling molecule with functions outside of the nervous system [11, 12]. That GABA may also be involved in the pathogenesis of several malignancies is only beginning to come to light. This report represents the first demonstration of the expression of GABA receptor subunits and GABARAP in thyroid cancer.

The rationale for this study was based upon previous gene expression profiling results that suggested that GABAergic system genes were up regulated in thyroid tumors compared to normal thyroid tissue. Our immunohistochemical studies have validated at the protein level that this is indeed the case. However, our results also present a

cautionary tale in over-interpreting the results of array-based expression analysis without further histopathological confirmation. The expression of $GABA_A$ β2 mRNA was increased in thyroid cancer by 30 fold by micro-array data; however, as shown by IHC, $GABA_A$ β2 protein was localized to blood vessels rather than epithelial cells in most cases. These findings were confirmed by *in vitro* study demonstrating absence of $GABA_A$ β2 expression in thyroid cancer cell lines. This is the first example of GABA receptor expression localized to blood vessels and suggests that GABA signaling may play a role in angiogenesis in thyroid cancer. The possible role of this receptor in the regulation of angiogenesis in other malignancies remains to be established.

In contrast to $GABA_A$ β2 expression, $GABA_A$ α2, $GABA_B$ R2 and GABARAP were expressed in thyroid cancer cells but not in the vasculature.

High levels of $GABA_A$ α2 expression were detected only in a small subset of thyroid tumors making clinical interpretation of these findings difficult. However, in vitro data showing expression of $GABA_A$ α2 in metastatic derived cell lines but not in primary-tumor derived cells suggests its possible role in the development of metastases. Studies examining this role are in progress.

$GABA_B$ R2 was differentially expressed between normal, benign, and malignant thyroid lesions, with normal tissue having undetectable expression, and malignant cancers having the highest expression and benign tumors displaying an intermediate level. The development of thyroid cancer is thought to be a multi-step process and our data demonstrating $GABA_B$ R2 in thyroid adenomas suggests the role of $GABA_B$ receptors at early stages of thyroid tumorigenesis.

GABARAP was also differentially expressed between normal, benign, and malignant thyroid lesions. GABARAP is an intracellular trafficking molecule that is responsible for the transport of GABA A receptor subunits to the cell membrane and anchoring them to the cytoskeleton. Recent publications have also suggested that GABARAP is an essential component of autophagic vacuoles [26–28]. Autophagy has emerged as one of the mechanisms involved in carcinogenesis, specifically in cancer cell resistance to chemotherapy. Our data showed that GABARAP is upregulated during thyroid tumorigenesis and therefore suggests a potential role for autophagy in thyroid carcinogenesis.

This study represents a retrospective analysis of the GABA system in thyroid lesions using archival paraffin embedded tissue and the thyroid hormone status of the patients included in this study was not available. Future functional studies to evaluate the effects of thyroid hormone on the GABA system in thyroid lesions are needed.

In conclusion, this study represents the first demonstration of GABA receptor expression in human thyroid tissue. It suggests that the GABAergic system is involved in thyroid carcinogenesis and future studies examining the functional role of the GABAergic system in thyroid cancer are needed. Additionally, the potential to use the many available GABA agents as adjuvant therapy for this disease needs to be investigated

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The authors have no conflicts of interest to disclose.

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