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The expression of GST and CYP isoenzymes in thyroid nodular hyperplasia and papillary thyroid cancer tissue: Correlation with clinical parameters

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ABSTRACT

This study investigated the immunohistochemical staining characteristics of glutathione-S-transferase (GST) pi(P), mu(M), theta(T), omega(O) and kappa(K) cytochrome P450 (CYP) A1, B1 and 2E1 isoenzymes in thyroid nodular hyperplasia (NH) and papillary thyroid cancer (PTC) tissues. For immunohistochemical studies, tissues from 18 patients with thyroid nodular hyperplasia, 28 patients with papillary thyroid cancer at the Kecioren Training and Research Hospital, Ankara, Turkey, were used. Relationships between GST and CYP isoenzyme expressions in NH and PTC tissues were examined by the Mann-Whitney U test, and clinicopathological data were examined by the Pearson Correlation Test and Regression Analysis. When the NH and PTC tissues from these cases were compared with respect to their staining intensity, GSTP1, GSTO1, GSTK1, CYP1A1, CYP2E1 expressions in PTC cells were significantly higher than those in NH epithelial cells ($p<0.05$). There were no statistically significant differences in the CYP1B1, GSTT1 and GSTM1 expressions between benign and tumor epithelium ($p>0.05$). There were significant association between GSTO1, GSTK1 expressions and sT3 levels in PTC ($p<0.05$) and CYP1B1 expression in NH. There was a significant association between GSTO1 expression and smoking status in NH ($p<0.05$). There was no statistical relationship between the GSTM1, GSTT1, GSTP1, GSTO1, GSTK1, CYP1A1, CYP1B1, CYP2E1 isoenzyme expressions and the clinicopathological data (age, TSH, sT4 levels, tumor stage) ($p>0.05$). GSTP1, GSTO1, GSTK1, CYP1A1 and CYP2E1 isoenzymes may have roles in the carcinogenesis of the papillary thyroid cancer.

Keywords: thyroid nodular hyperplasia, papillary thyroid cancer, glutathione-S-transferase, cytochrome P450 enzymes, immunohistochemistry

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and its incidence has been growing steadily [1]. Thyroid cancer occurs in 5–10% depending on age, gender, radiation exposure history, family history, and other factors [1].

Although the etiology of thyroid cancer is still unknown, exposure to ionizing radiation, dietary iodine deficiency are the cause of thyroid carcinogenesis in humans [2-4]. However, individuals without previous exposure to ionizing radiation can also develop thyroid cancers [5], suggesting that other risk factors could also be involved in the etiology of thyroid cancers.

The expression of both phase I (cytochoromes P450 (CYP)) and phase II (glutathione-S-transferases (GST)) enzymes in the target organ cells can be important in determining the occurence of carcinogenesis as related to

carcinogen exposure. Among the human CYP enzymes examined, CYP1A1, 1A2, 2E1 and 3A4 are generally recognized to be the major forms involved in the activation of most of the procarcinogens in human liver and lung microsomes [6]. The GST family includes phase II enzymes that detoxify carcinogens and reactive oxygen species [7]. The GST family has been assigned to eight distinct classes: GSTA, GSTM, GSTT, GSTP, GSTS, GSTK, GSTO, and GSTZ [8, 9].

The carcinogen metabolizing enzymes are involved in the activation and deactivation of diverse chemical carcinogens. Inter-individual and inter-racial variations in the expression of these CYP and GST enzymes in target tissues may explain the differences in susceptibility observed in clinical and epidemiological studies [10].

In this study, we assessed the cellular prevalence and distribution of GSTP, GSTM1, GSTO1, GSTK1, GSTT1, CYP1A1, CYP1B1 and CYP2E1 isoenzymes in nodular hyperplasia (NH) and papillary thyroid carcinoma (PTC) tissues. The statistical analysis were studied between the patients' clinical parametres (age, gender, smoking status, TSH, sT3, sT4 levels, tumor stage) and isoenzymes expressions.

MATERIALS AND METHODS

Patients

NH (n=28) and TPC (n=18) tissue samples were taken from 40 male and 6 female patients (46 in total) who were diagnosed at Kecioren Training and Research Hospital, Ankara, Turkey, between 2009-2012, were stained immunohistochemically with the antibodies. For all patients, total serum sT3, sT4, TSH levels, tumor stage, patient age, were known. Operation material was examined macroscopically by two pathologists in each case. Tissues were fixed overnight. Two sections were taken from each patient: one from the tumor tissue and one from the macroscopically normal tissue peripheral to the tumor tissue.

Immunohistochemical staining

The tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. Sections 4 µm thick were cut, and one section was stained with hematoxylin and eosin to observe the tissue morphology and tumor score. For immunohistochemistry, endogenous endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide (v/v) in methanol for 10 minutes at room temperature (RT). The sections were subsequently washed in distilled water for 5 minutes, and antigen retrieval was performed for 3 minutes using 0.01M citrate buffer (pH 6.0) in a domestic pressure cooker. The sections were transferred in 0.05M Tris-HCl (pH 7.6) containing 0.15M sodium chloride (TBS). After washing in water, the sections were incubated at RT for 10 minutes with super block (SHP125) (ScyTek Laboratories, USA) to block nonspecific background staining. The sections were then covered with the primary antibodies diluted 1:500 for anti-GSTP1, 1:500 for anti-GSTK1, 1:100 for anti-GSTM1, 1:500 for anti-GSTT1, 1:400 for anti-GSTO1, 1:50 for anti-CYP1A1, 1: 300 for anti-CYP1B1, 1:300 for anti-CYP2E1 in TBS at 4^{o}C overnight (Anti-GSTK1 (EPR1939) was from Origene Technologies Inc., USA; GSTM1 (ab113432) and GSTT1 (ab96592) were from Abcam Inc., USA; GSTO1 (ab88604) was from Abcam Inc., USA; Anti-CYP1A1 (sc-20772) and Anti-GSTP1 (sc-28494) were from Santa Cruz Biotechnology Inc., USA; Anti-CYP1B1 (sc-32882) was from Santa Cruz Biotechnology Inc., USA; Anti-CYP2E1 (PA1116) was from BOSTER Biological Technology., Ltd. USA). After washing in TBS for 15 minutes, the sections were incubated at RT for biotinylated link antibody (SHP125) (ScyTek Laboratories, USA). Then, treatment was followed with Streptavidin/HRP complex (SHP125) (ScyTek Laboratories, USA). Diaminobenzidine was used to visualise peroxidase activity in the tissues. Nuclei were lightly counterstained with haemotoxyline, and then the sections were dehydrated and mounted. Both positive and negative controls were included in each run. Positive controls consisted of sections of liver tissues for GSTP1, GSTK1, GSTM1, lung tissues for GSTT1, CYP2E1, colon tissues for GSTO1, gall bladder tissues for CYP1A1 and skeletal muscle tissues for CYP1B1. TBS was used in place of the primary antibody for negative controls.

Light microscopy of immunohistochemically stained sections was performed by a pathologist and a biologist, who were unaware of the patients' clinical information. Distribution, localization and characteristics of immunostaining were recorded. Brown colour in cytoplasm and/or nucleus of epithelial cells of the thyroid tissue was evaluated as positive staining. Scoring was also performed by observers unaware of the patients' clinical information. Scoring differences between observers were resolved by consensus. For each antibody, the intensity of the reaction—negative (-), weak (1+), moderate (2+) or strong (3+)—was determined in order to describe the immunoreactions.

Statistical Analysis

In the study, MINITAB 14 statistical software (MINITAB®release 14.12.0, MINITAB Inc., State Collage, Pennsylvania,United States) was used for statistical evaluations. In the study and control group tissues, the differences between protein expressions were searched by Pearson correlation test with 95% accuracy, to investigate

the relations between clinical data, such as Mann–Whitney U-test; CYP1A1, CYP1B1, CYP2E1, GSTO1, GSTK1, GSTP1 and GSTT1 expressions of patients' thyroid nodular hyperplasia, papillary thyroid cancer tissues and their ages, gender, and smoking habits. The results were found to be significant for $p < 0.05$.

RESULTS AND DISCUSSION

The median patient age was 52 years (minimum: 17, maximum: 81), the normal value of patient's TSH levels was 0.3-3.6 mlU/I; the normal value of sT3 was 2.2- 4.2 pg/mL; the normal value of sT4 was 0,65-1,7 ng/dI. Scoring for each patient's TSH, sT3, sT4 levels was performed as (1) for lower than normal values, (2) for normal values, (3) for higher than normal values. Ten cases were stage I, 4 cases were stage II, and 4 cases were stage III papillary thyroid carcinoma.

Papillary thyroid carcinoma from twenty-eight patients and thyroid nodullar hyperplasia tissues from 18 patients were examined. The CYP1A1 (100%), CYP1B1 (55.56%), CYP2E1 (72.22%) expressions were higher in PTC epithelium than that in NH epithelium in thyroid tissues (Table 1) (Fig.1).

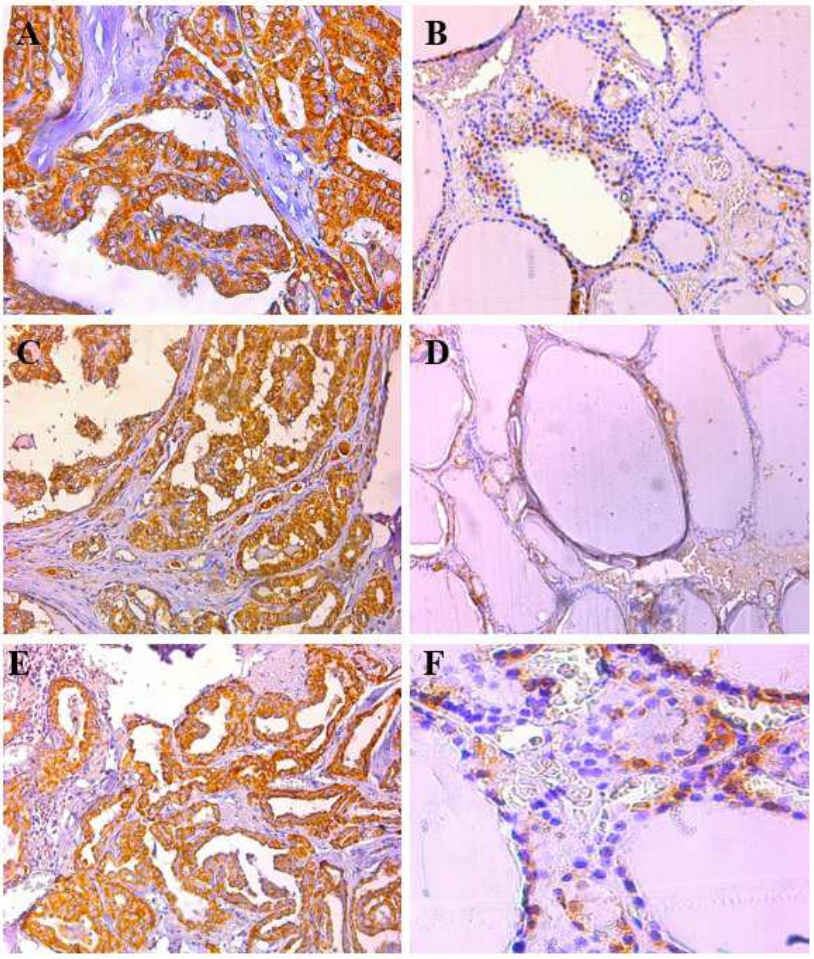

Figure 1. Immunohistochemical expression of GST isoenzymes in patients with papillary thyroid cancer. A: strong (+3) GSTP1 expression in papillary thyroid carcinoma (x200), B: weak (+1) GSTP1 expression in nodular hyperplasia (x100), C: strong (+3) GSTO1 expression in papillary thyroid carcinoma (x200), D: weak (+1) GSTO1 expression in nodular hyperplasia (x100), E: strong (+3) GSTK1 expression in papillary thyroid carcinoma (x200), F: weak (+1) GSTK1 expression in nodular hyperplasia (x200).

Table 1. The number and percentage of patients with Nodullar Hyperplasia and Papillary Thyroid Carcinoma that seen CYP isoenzymes expressions

	CYP1A1	CYP1B1	CYP2E1
	n/%n[c]	n/%n	n/%n
NH[a] (n=28)	23/82.14 (1-3)[d]	10/35.71 (1-3)	9/32.14 (1-2)
PTC[b] (n=18)	18/100 (1-3)	10/55.56 (1-3)	13/72.22 (1-2)

a: Nodular Hyperplasia,b:Papillary thyroid carcinomac: Percentages are by rows in positively stained cells.d: min. and max. Staining intensity

CYP1A1 and CYP2E1 expressions were statistically higher in PTC than NH thyroid tissues (p=0.0003, p=0.023; respectively) but, there was no statistically differences in CYP1B1 isoenzyme expression (p> 0.05) (Table 2).

Table 2. Statistically Differences of CYP isoenzymes expressions between patients with Nodullar Hyperplasia and Papillary Thyroid Carcinoma

	CYP1A1	CYP1B1	CYP2E1
PTC[a] (n=18)	2.33±0.21[e] (1-3)[f]	0.94±0.24 (0-3)	0.89±0.16 (0-2)
NH[b] (n=28)	1.14±0.14 (0-3)	0.64±0.19 (0-3)	0.39±0.12 (0-2)
PTC/NH[c]	2.04	1.47	2.28
***p* value[d]**	***0.0003***	0.2953	***0.023***

Differences of CYP isoenzymes expression between patients with Nodullar Hyperplasia and Papillary Thyroid Carcinoma were examined by the Mann-Whitney U test with 95% confidence level.

a: Papillary thyroid carcinoma
b: Nodular Hyperplasia
c: Rate of PTC and NH
d: p value less than 0.05 was considered statistically significant.
e: Mean±Standart Error Mean
f: min. and max. Staining intensity

According to the GST isoenzymes immunohistochemical staning results, GSTP1(100%), GSTM1(83.3%), GSTO1(94.44%) and GSTK1(88.88%) expressions were higher in PTC epithelium than that in NH epithelium in thyroid tissues. However, GSTT1(100%) expression was higher in NH epithelium than PTC (Table 3) (Fig.2).

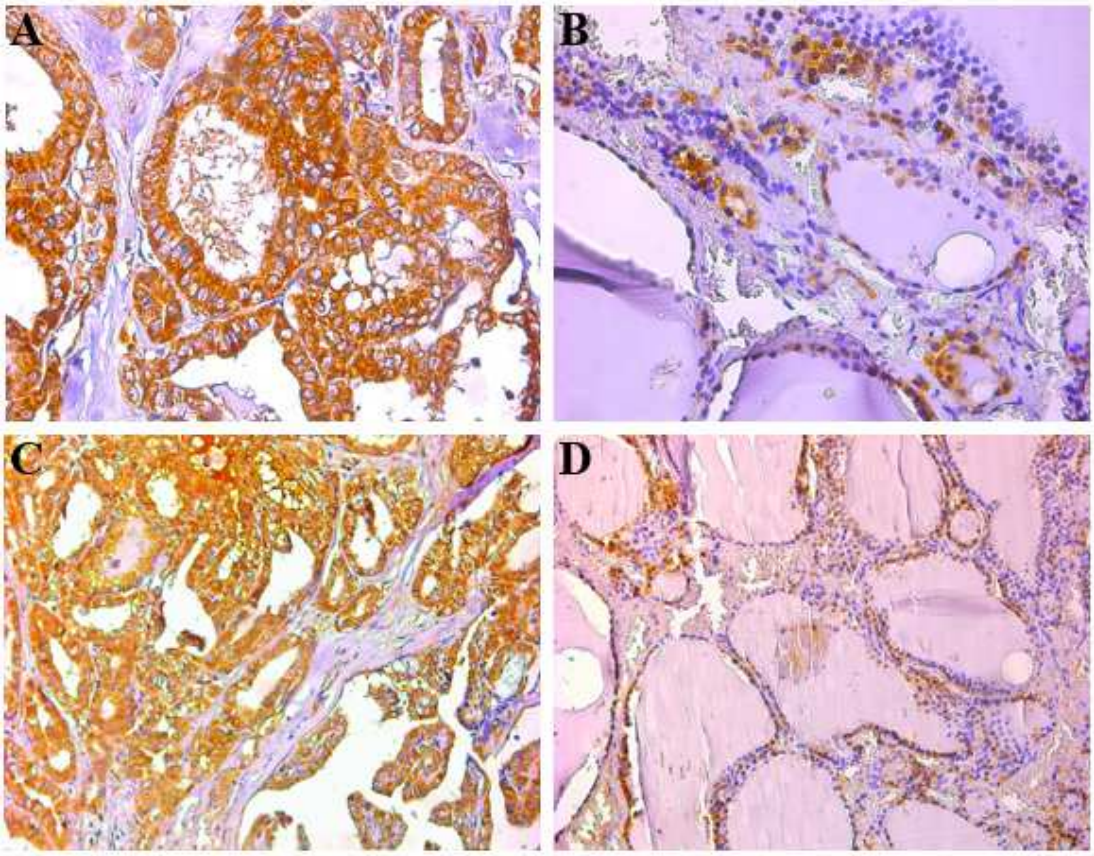

Figure 2. Immunohistochemical expression of CYP isoenzymes in patients with papillary thyroid cancer. A: strong (+3) CYP1A1 expression in papillary thyroid carcinoma (x200), B: weak (+1) CYP1A1 expression in nodular hyperplasia (200X), C: strong (+3) CYP2E1 expression in papillary thyroid carcinoma (x200), D: weak (+1) CYP2E1 expression in nodular hyperplasia (x100).

Table 3. The number and percentage of patients with Nodullar Hyperplasia and Papillary Thyroid Carcinoma that seen GST isoenzymes expressions

	GSTP1	GSTM1	GSTT1	GSTO1	GSTK1
	n/%n[c]	n/%n	n/%n	n/%n	n/%n
NH (n=28)[a]	27/96,42 (1-3)[d]	24/85,71 (1-2)	28/100 (1-3)	23/82,14 (1-3)	13/46,42 (1-2)
PTC (n=18)[b]	18/100 (2-3)	15/83,33 (1-3)	15/83,33 (1-3)	17/94,44 (1-3)	16/88,88 (1-3)

a*: Nodular Hyperplasia,b:Papillary thyroid carcinomac: Percentages are by rows in positively stained cells.d: min. and max. Staining intensity*

GSTP1, GSTK1 and GSTO1 expressions were statistically higher in PTC than NH thyroid tissues (p=0.0047, p=0.0001, p=0.0001; respectively) but, there was no statistically differences in GSTM1 and GSTT1 isoenzyme expression (p> 0.05) (Table 4).

Table 4. Statistically Differences of GST isoenzymes expressions between patients with Nodullar Hyperplasia and Papillary Thyroid Carcinoma

	GSTP1	GSTM1	GSTT1	GSTO1	GSTK1
PTC [a] (n=18)	2,83±0,09 (2-3)	1,67±0,23 (0-3)	2,22±0,29 (0-3)	2,56±0,22 (0-3)	1,94±0,25 (0-3)
NH [b] (n=28)	2,18±0,15 (0-3)	1,25±0,13 (0-2)	2,68±0,12 (1-3)	1,18±0,16 (0-3)	0,5±0,11 (0-2)
PTC/NH[c]	1,30	1,34	0,83	2,17	3,88
***p* value[d]**	**0,0047**	0,1076	0,4308	**0,0001**	**0,0001**

Differences of GST isoenzymes expression between patients with Nodullar Hyperplasia and Papillary Thyroid Carcinoma were examined by the Mann-Whitney U test with 95% confidence level.

a: Papillary thyroid carcinoma
b: Nodular Hyperplasia
c: Rate of PTC and NH
d: p value less than 0.05 was considered statistically significant.
e: Mean±Standart Error Mean
f: min. and max. Staining intensity

There was a statistically significant relationship between CYP1A1, CYP1B1 and GSTM1 expressions in PTC patients (p<0.05) however, there were no relationships between isoenzymes in NH patients (p>0.05).

The clinical and pathologic characteristics of the PTC cancers and the levels of GSTP1, GSTM1, GSTT1, GSTO1, GSTK1 and CYP1A1, CYP1B1, CYP2E1, expressions were correlated separately. There was a significant negative correlation between sT3 level of patients and GSTO1 and GSTK1 expression in PTC tissues (r=-0.495; p=0.037 respectively), CYP1B1 expression in NH tissues (r=-0.512; p=0.030 respectively). There was a significant positive correlation between smoking status and GSTO1 expression in NH tissues. There was no statistical relationship between GSTM1, GSTT1, GSTP1, GSTO1, GSTK1, CYP1A1, CYP1B1, CYP2E1 isoenzyme expressions and the clinicopathological data (age, TSH, sT4 levels, tumor stage) (p > 0.05).

In this study, we investigated the association of neoplastic transformation in thyroid tissue and the protein expression of CYP and GST isoenzymes. CYP enzymes oxidize cytotoxic and carcinogenic agents, yielding reactive epoxide intermediates, which can covalently bind and alter DNA structure [11]. GST enzymes catalyze glutathione (GSH) conjugation of these intermediates, thereby decreasing their DNA-damaging effects [12]. Variations in the expression of CYPs and GST could potentially explain the observed difference in vulnerability to the carcinogenic effects of these carcinogens. The alpha, mu, pi, and theta subclasses are mostly expressed in mammalian tissues, with GSTP (the major class of the GSTs) being the most abundant in the urinary, respiratory, gastrointestinal systems and thyroid [13,14]. GSTP protein plays a role in toxin excretion and metabolism. Loss of GSTP function may render thyroid cells vulnerable to genome damage mediated by environmental carcinogens that may be GSTP substrates, including oxidants, such as those arising from thyroid inflammation, and electrophiles, which may be contributed via dietary exposure to heterocyclic aromatic amine carcinogens [15].

Tissue metabolism of carcinogens by these locally expressed enzymes may be a more important determinant of carcinogenesis than metabolism in the more distant organs, such as the liver. Several studies assessed the risk of thyroid cancer in relation to CYP and GST genotypes. Reis *et al.* [16] found no significant association of CYP1A1

null genotype with thyroid diseases but they showed that the null genotypes of GSTM1 and GSTT1 genes were predominant in patients with nodules. Bufalo *et al*. [17] showed that CYP1A1 null genotype might be associated with reduced risk to papillary thyroid carcinomas among smokers. Morari *et al*. [18] suggested that GSTT1 and GSTM1 null genotypes were associated with an increased susceptibility to thyroid cancer. Siraj *et al.* [19] found no relationship between the risk of thyroid carcinoma and CYP1A1, GSTP1 enzymes, but they indicated significant risk of developing thyroid cancer compared to GSTT1 and GSTM1 enzymes. Gaspar *et al*. [20] showed that GSTM1 null, GSTT1 null, and GSTP1 Ile/Ile polymorphisms lead to a moderate increased risk for thyroid papillary cancer. Hernández *et al.* [21] and Kweon et al. [22] did not find any association between the polymorphism at GSTM1, GSTT1 and GSTP1 genes and thyroid cancer incidence. Morari *et al*. [23] and Granja *et al*. [24, 25] suggested that GSTT1, GSTM1, GSTP1, but not GSTO1, increased the risk of thyroid cancer.

Interindividual variations in the expression and activity of GST are dependent on genotypic and posttranscriptional factors, which may be tissue specific [26-28]. Therefore, the most direct method to evaluate the effects of GST on the carcinogenic effects of tobacco is to evaluate the enzyme expression in the tissue of interest. To our knowledge, the present study represents the first comprehensive description of the three classes of CYPs and five classes of GSTs in thyroid nodular hyperplasia and papillary thyroid cancer tissues. We observed higher staining intensity for GSTP1, GSTO1, GSTK1, CYP1A1 and CYP2E1 isoenzymes in tumor epithelial cells compared with nodular hyperplasia cells ($p<0.05$). However, there was no statistically significant differences in CYP1B1, GSTM1 and GSTT1 isoenzyme expression ($p> 0.05$). Induction of the enzymes in thyroid cancers could be an adaptive response to stress or to chemical agents. Cancer cells reveal multiple genetic alterations resulting in morphologic and functional differences from normal cells. Tumour cells may lose some of their functions (eg, expression of some proteins) in the malignant transformation process. It can be hypothesized that higher levels of GST and CYP expressions in tumour cells are the result of this transformation.

CONCLUSION

These results suggest that the GST, CYP population, owing to higher expression of multiple GSTs, CYPs especially GSTP1, GSTO1, GSTK1 CYP1A1 and CYP2E1, can play a role in tumor growth and carcinogenesis of the papillary thyroid cancer.

This study demonstrates the wide variability in GST and CYP enzymes expression in papillary thyroid cancer. Incorporating such an approach in larger trials may help elucidate the roles of these enzymes in carcinogenesis and identify potential targets for chemoprevention.

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