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ORIGINAL ARTICLE

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Enzyme expression profiles suggest the novel tumor-activated fluoropyrimidine carbamate capecitabine (Xeloda) might be effective against papillary thyroid cancers of children and young adults

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Abstract *Purpose*: The fluoropyrimidine carbamate (capecitabine) is converted to 5-fluorouracil (5-FU) by thymidine phosphorylase (TP) inside target tissues. 5-FU interferes with DNA synthesis by blocking thymidylate synthase (TS) but is inactivated by dihydropyrimidine dehydrogenase (DPD). Favorable enzyme profiles (high TP and low DPD) generate high intratumor levels of 5-FU that are effective against many tumors, especially those with low TS. Capecitabine has not been tested against thyroid cancers, and it is not known to what extent thyroid cancers express TP, TS or DPD. *Methods*: To test this, we determined TP, TS and DPD in 19 thyroid cancers from young patients (14 papillary, 4 follicular, 1 medullary) by immunohistochemistry. After approval by the Human Use Committee, the intensity of TP, TS, and DPD staining was determined by two independent examiners and graded (absent = 0 to intense = 3) with >90% concordance. *Results*: TS was detected in 7/19 cancers (37%), TP in 14/19 cancers (74%) and DPD in 14/19 cancers (74%). In six tumors, TP was more intense that DPD, suggesting capecitabine sensitivity. Only five tumors failed to express TP but four of these expressed DPD, suggesting capecitabine resistance. Overall, 6/19 tumors (32% of the total) had a favorable expression profile, and all of them were papillary cancers. *Conclusions*: We conclude that the majority of differentiated thyroid cancers (74%) express TP and low levels of TS (63% undetectable). The results support the hypothesis that capecitabine is activated in the majority of differentiated thyroid cancers and that 32% have favorable expression of all three enzymes (TP, TS, and DPD).

Keywords Thyroid cancer · Drug treatment · Capecitabine

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Introduction

Thyroid carcinomas are the most common endocrine cancers in adults and children [6, 22]. Most are well differentiated and respond to surgery and radioactive iodine (^{131}I), but many persist, dedifferentiate, or undergo anaplastic transformation, resulting in more deaths than cervical or testicular cancers [6, 22]. Anaplastic thyroid cancer is among the most aggressive human cancers, and displays relative resistance to chemotherapy [14, 17, 19, 21]. The mechanism of drug resistance is unknown. Expression of the multiple drug resistance 1 (MDR-1) gene is low and unrelated to drug sensitivity [13]. Hyperfractionated external beam radiation, doxorubicin, or bleomycin, cytoxan, and 5-fluorouracil (5-FU) have induced limited remissions [18, 19, 20], and a more recent study suggests that paclitaxel (Taxol) may have some effect [1]. Based on this limited success, there is a need for additional

therapeutic agents in the treatment of thyroid cancer [15].

The novel, tumor-activated fluoropyrimidine carbamate (capecitabine, Xeloda; Hoffman-LaRoche, Indianapolis, Ind.) is well absorbed after oral administration, and converted inside the target tissues into 5-FU by thymidine phosphorylase (TP) [2, 3, 4, 5]. 5-FU interferes with DNA synthesis by blocking thymidylate synthase (TS) but is inactivated by dihydropyrimidine dehydrogenase (DPD) [10, 11, 12, 16]. Favorable enzyme profiles would have high TP and low DPD and be effective against many tumors, especially those with low TS [10, 11, 12, 16]. Because TP is generally present at higher levels in malignant tissues, Capecitabine generates high intratumor levels of 5-FU with limited exposure of normal tissues [10, 11, 12, 16]. Our goal was to determine if the expression profiles of the enzymes that metabolize capecitabine might be favorable for capecitabine treatment of thyroid cancers.

Materials and methods

Approval

Prior approval and funding for this study (WU# 01-65001B) were obtained from the Human Use Committee and the Department of Clinical Investigation, Walter Reed Army Medical Center, Washington, DC.

Patients

We had previously searched the automated centralized tumor registry of the Department of Defense (ACTUR) to identify all patients diagnosed with thyroid cancer prior to 21 years of age [22]. The extent of disease at diagnosis was classified according to the system of DeGroot et al. [6] and the metastasis, age, completeness of resection, invasion, and size (MACIS) scoring system [9]. In the classification of DeGroot et al., class 1 disease is confined to the thyroid, class 2 involves the regional lymph nodes, class 3 extends beyond the capsule or is inadequately resected, and class 4 has distant metastasis. All patients <39 years of age have a MACIS score calculated as $3.1 + (\text{size} \times 0.3) + 1$ (if incomplete resection) + 1 (if extension beyond the capsule) + 3 (if distant metastasis) [9]. Recurrence was defined as the appearance of new disease (identified by ^{131}I scan or biopsy) in any patient who had been free of disease (no disease palpable or identified by ^{131}I scan) for at least 4 months after initial therapy [22]. The majority of patients received their medical care prior to the routine use of serum thyroglobulin (Tg) levels for defining recurrence-free survival. For this reason, Tg levels are not used in our definitions. However, Tg levels were determined for contemporary patients (normal 3–40 ng/ml; University of Southern California Clinical Laboratories, Los Angeles, Calif.). Sufficient formalin-fixed paraffin-embedded archival tumor tissue was available to stain 14 papillary thyroid cancers (PTC), 4 follicular thyroid cancers (FTC), and 1 medullary thyroid cancer (MTC) for TP, TS and DPD by specific immunohistochemistry.

Immunohistochemistry

Sections were stained with hematoxylin and eosin to confirm the diagnosis, and sections immediately adjacent (5 μm) were deparaffinized with xylene and rehydrated [8]. Antigens were retrieved using Declere (Cell Marque, Hot Springs, Ariz.) and endogenous peroxidase was quenched (3% H_2O_2, 30 min, room temperature). Nonspecific binding was blocked (20% normal goat serum) and the sections were incubated with primary anti-TP (1:25, Neomarkers, Fremont, Calif.), anti-TS (1:50, Roche, Indianapolis, Ind.), or anti-DPD (1:50, Roche), followed by biotinylated secondary anti-rat antibody (1:100, Z-0455, Dako, Carpinteria, Calif.). Sections were stained on a Ventana automated slide stainer (NEXES, Tucson, Ariz.) using Ventana DAB detection and amplification kits, followed by hematoxylin counterstaining and bluing. The presence and intensity of each stain was quantified from absent (grade 0) to intense (grade 3) by two independent blinded examiners with $>90\%$ concordance. Human breast cancer was used as the positive control, and phosphate-buffered saline was substituted for the primary or secondary antibodies as negative controls.

In vitro detection of TS, TP and DPD in thyroid cancer cell lines

The mRNAs encoding TS, TP and DPD were amplified from thyroid cancer cell lines by reverse transcription/polymerase chain reaction (RT/PCR). Moderately differentiated PTC (NPA), poorly differentiated FTC (WRO) and anaplastic thyroid cancer (ARO-81) cells were a generous gift from Dr. G. Juillard (University of California, Los Angeles, Calif.) [7]. Samples were treated with DNAse (Ambion, Austin, Tx.), total RNA was extracted (Trire-agent, 1 ml, 0°C) and 1 μg was reverse transcribed using random $(dNTP)_6$ and the Impromptu RT kit (Promega, Madison, Wis.) at 42°C for 1 h, followed by 15 min at 70°C. PCR was performed in optimized PCR buffer (25 μl) containing enhancer and stabilizer, along with dNTPs (Maxim Biotech), equal amounts of sense and antisense primers (10 pmol of each) and Platinum Taq polymerase (0.625 U; Invitrogen, Carlsbad, Calif.). Parameters for TP and DPD included an initial denaturation at 96°C for 1 min, an annealing step at 66°C (TP) or 62°C (DPD) for 4 min, 28 amplification cycles at 94°C for 1 min, and the respective annealing temperatures for TP and DPD, followed by a final extension at 72°C for 7 min. For TS, denaturation was at 95°C for 2 min, followed by 30 amplification cycles at 95°C for 2 min, annealing at 58°C and a 1-min final extension. RT negative controls, and expression of the internal housekeeping gene, glyceraldehyde-3-PO_4 dehydrogenase (GAPDH) were included in each reaction. PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. The primer sequences are shown in Table 1.

Data analysis and statistical comparisons

The presence and intensity of TS, TP and DPD staining were correlated with the histologic variant, demographic features, extent of disease at diagnosis, and clinical outcome. Statistical analyses were performed using SPSS for Windows 95 (version 7.5; SPSS, Chicago, Ill.). Average staining intensities were compared using ANOVA.

Results

The clinical features of the 19 patients, stratified according to the expression profiles of TS, TP and DPD, are shown in Table 2. They ranged in age from 6 to 21 years (mean 17.6 ± 3.8 years) and had tumors with an average size of 1.9 ± 1.4 cm (range 0.2–5.5 cm). Seven patients (7/14, 50%) had class 1 PTC, six (6/14, 43%) had class 2 PTC, and one (1/14, 7%) had class 4 PTC. The average MACIS score for those with PTC was 3.92 ± 1.07 (range 3.31–7.39). None of the patients had

Table 1 Primer sequences for TP, TS and DPD PCR amplification

	Sense	Antisense	Size (bp)
TP	TGGCTCAGTCGGGACAGCAG	TCCGCTGATCATTGGCACCT	152
TS	GAATCACATCGAGCCACTGAAA	GTGTTACTCAGCTCCCTCAGA	579
DPD	TCCTCCAGGTATGCAGTGCCA	GTTATGGTGGGCAGGTGGGTT	506
GAPDH	obtained from Maxim Biotech (San Francisco, Calif.)		

Table 2 Clinical features and enzyme expression profiles

TP/DPD expression profile	Patient	Age (years)	Gender	Histology	Class[a]	Tumor size (cm)	MACIS score[b]	Focal (uni/multi)[c]	Surgery[d]	Node surgery[e]	^{131}I	Follow up (months)	Recurrence (months)	TS	TP	DPD
Favorable[f]	1	20	F	PTC	1	0.7	3.31	Uni	Total	Select	Y	39		0	2	0
	2	21	F	PTC	1	1.2	3.46	Multi	Total	Select	Y	107	12	1	3	2
	3	21	F	PTC	1	1.8	3.64	Uni	Total	Select	N	68		0	3	0
	4	17	F	PTC	1	2.4	3.82	Uni	Total	MR	Y	50		0	1	0
	5	20	F	PTC	2	1.0	3.4	Uni	Total	MR	Y	101		2	3	0
	6	14	F	PTC	2	3.0	4.0	Multi	Total	MR	Y	86	67	0	3	2
Indeterminate[g]	7	20	M	PTC	1	1.2	3.46	Uni	Lobe	Select	N	118		0	1	2
	8	16	F	PTC	1	1.5	3.55	Multi			Y	19		0	2	2
	9	15	F	PTC	2	0.7	3.31	Multi	Total	MR	Y	85		1	1	2
	10	19	M	PTC	2	1.0	3.4	Multi				104		0	1	2
	11	21	F	PTC	2	1.0	3.4	Multi	Total	MR		22		0	3	3
	12	13	F	PTC	2	5.5	4.75	Multi	Lobe			0		1	1	2
	13	6	F	PTC	4	4.3	7.39	Multi	Sub	MR	Y	169	6	1	1	2
	14	17	F	FTC		2.2		Multi	Total	Select	Y	33	9	0	2	2
Unfavorable[h]	15	18	F	PTC	1	2.9	3.97	Uni	Total	Select	Y	100		0	0	0
	16	20	F	FTC		0.2		Uni	Total	Select	Y	34		0	0	4
	17	17	F	FTC		2.5		Uni	Total	Select	Y	43		2	0	1
	18			FTC										1	0	4
	19	21	M	MTC		0.7		Multi	Total	Rad	N	59		0	0	1

[a]Class refers to the system of DeGroot et al. and is only used for PTC [6]
[b]MACIS refers to the system of Hay et al. and is only used for PTC [9]
[c]Focal refers to presence of unifocal or multifocal disease at pathology
[d]Surgery refers to the first operation including ''completion'' thyroidectomy (*Total* total thyroidectomy, *Sub* subtotal thyroidectomy, *Lobe* lobectomy)
[e]Node surgery refers to type of initial node dissection (*Select* zremoval of suspicious nodes, *MR* modified radical,*Rad* radical dissection)
[f]Favorable profile: intensity of TP greater than that of DPD
[g]Indeterminate profile: TP expressed but intensity less than that of DPD
[h]Unfavorable profile: TP not detected

previous radiation exposure. The patients were followed for an average of 69 ± 43 months (range 0–169 months). Four developed recurrent disease (4/19, 21%). The clinical details, treatment and outcome are similar to those of a larger cohort previously published by our group [22].

Fewer than half of the tumors stained for TS (7/19, 37%), but the majority stained for TP (14/19, 74%) and DPD (14/19, 74%) (Fig. 1). Sufficient normal thyroid was identified on a few slides ($n = 5$) to determine that TS and TP were undetectable (0/5), and that DPD was infrequently seen (2/6) in normal thyroid. For TP, this difference was statistically significant (14/19 thyroid cancers vs 0/5 normal thyroid, $P = 0.006$).

Six tumors (6/19, 32%) had favorable expression profiles (TP was more intense than DPD; Table 3) suggesting that they might respond to capecitabine. All six were PTC. Five tumors (5/19, 26%) failed to express TP (unfavorable profile). Four of these (4/5, 80%) also expressed DPD suggesting that they would be resistant to capecitabine (Table 3). Three of the five (3/5, 60%) were FTCs and 1 (1/5, 20%) was an MTC. The remaining eight tumors had indeterminate profiles in which TP was expressed, but the intensity was less than that of DPD (Table 3). Seven (7/8, 88%) were PTC and one was an FTC (1/8, 12%).

Overall, 6/14 (43%) PTC had favorable expression profiles suggesting they might respond to capecitabine therapy. In contrast, 3/4 (75%) FTC and the only MTC had unfavorable profiles suggesting resistance to capecitabine therapy. The difference between histologic variants, however, was not significant ($P = 0.58$, Fisher's Exact Test).

We also used RT/PCR to determine the expression of TS (Fig. 2A), TP (Fig. 2B) and DPD (Fig. 2C) in three moderately differentiated (NPA), poorly differentiated (WRO), and anaplastic thyroid cancer (ARO) cell lines. TS and DPD were highly expressed by all three, but TP

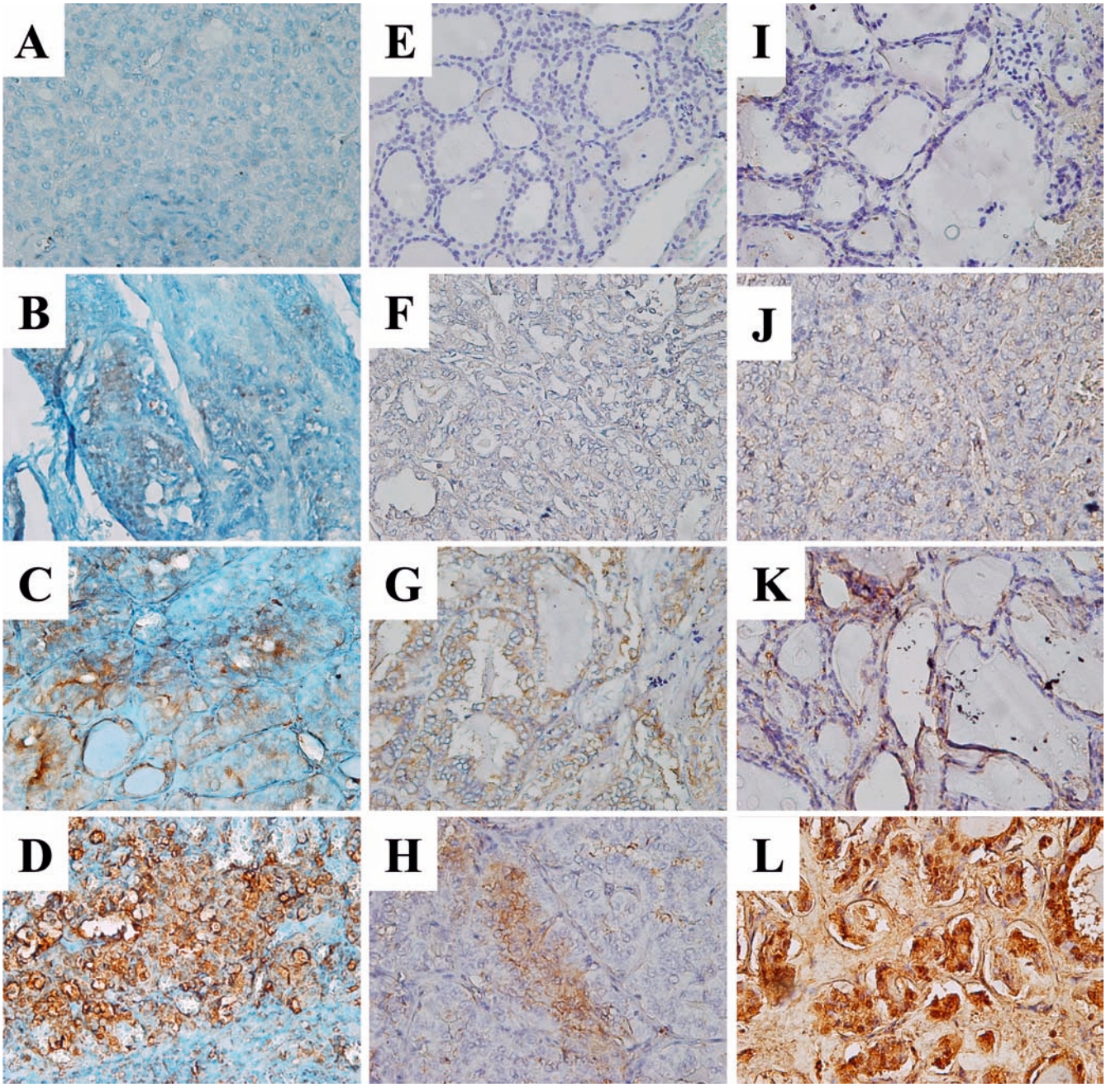

Fig. 1A–L Immunostaining for TP, TS and DPD. Representative sections are shown for TP (**A–D**), TS (**E–H**) and DPD (**I–L**) staining. The intensity of staining was graded as absent (grade 0 in sections **A**, **E**, **I**), minimal (grade 1 in sections **B**, **F**, **J**), moderate (grade 2 in **C**, **G**, **K**) and intense (**D**, **H**, **L**). All sections are shown at ×400 magnification

was only detected in the moderately differentiated PTC-derived (NPA) cells.

Discussion

The majority of thyroid cancers are well differentiated and respond to surgery and ^{131}I [22]. Unfortunately, about 20% recur and/or persist despite treatment [22]. For these patients, alternative therapies would be attractive if systemic toxicity was minimal and if there was a means by which to determine if the tumor was likely to respond.

Capecitabine has many features that suggest that it might be an acceptable agent for these patients. First, it is converted by TP into 5-FU in malignant tissues, limiting systemic toxicity [10, 11, 12, 16]. Second, all three of the critical enzymes involved in the metabolism of capecitabine can be identified by immunohistochemistry on formalin-fixed paraffin-embedded tissue, eliminating the requirement for enzymatic assays that can only be done on fresh tissue at the time of surgery.

Table 3 Thyroid cancers stratified according to TP/DPD expression profiles. Values are means ± SEM

	Favorable profile ($n=6$)[a]	Indeterminate profile ($n=8$)[b]	Unfavorable profile ($n=5$)[c]
Patient age (years)	18.8 ± 1.13	15.9 ± 1.7	19.0 ± 0.91
Tumor size (cm)	1.7 ± 0.36	2.2 ± 0.63	1.6 ± 0.66
MACIS score	3.61 ± 0.11	4.18 ± 0.57	3.97 ($n=1$)
Follow-up (months)	75 ± 11.2	69 ± 21	59 ± 14.6
Time to recurrence (months)	39.5 ± 39 ($n=2$)	7.5 ± 1.5 ($n=2$)	None
TS (mean intensity)	0.5 ± 0.34	0.38 ± 0.18	0.6 ± 0.40
TP (mean intensity)	2.5 ± 0.34	1.5 ± 0.27	0
DPD (mean intensity)	0.67 ± 0.42	2.12 ± 0.13	2.0 ± 0.84

[a]Favorable profile: TP staining intensity greater than that of DPD
[b]Indeterminate profile: TP present but intensity less than that of DPD
[c]Unfavorable profile: TP staining absent

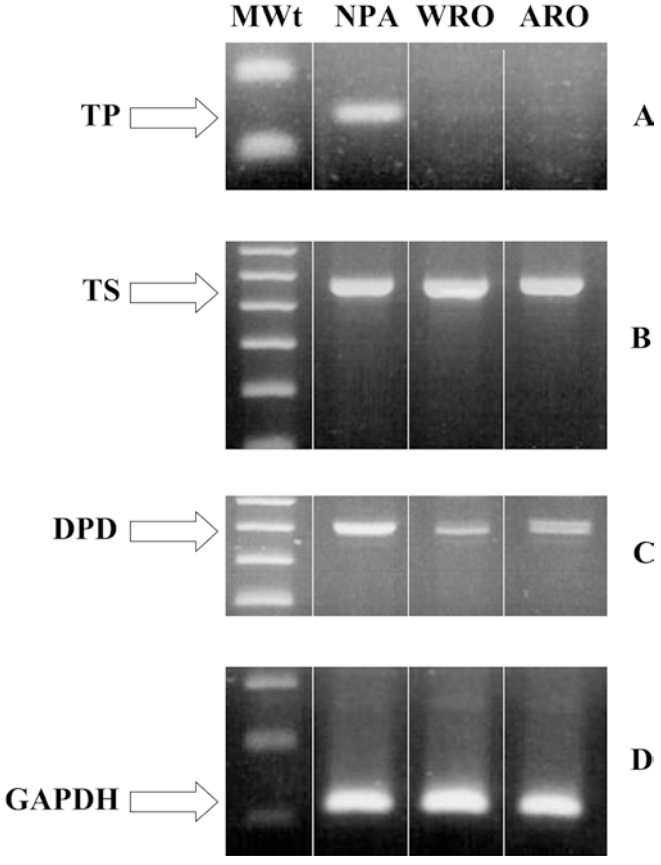

Fig. 2A–D RT-PCR amplification of TP, TS and DPD. The PTC-derived cell line (NPA) was grown in DMEM supplemented with 10% fetal bovine serum. Total RNA was extracted, reverse transcribed and specific sequences for TP (**A**), TS (**B**), DPD (**C**) and GAPDH (**D**) were amplified

We examined TS, TP and DPD expression in a group of thyroid cancers from children and young adults and found that TS could be detected in a minority of thyroid cancers (7/19, 37%). This would suggest that most thyroid cancers have low levels of TS rendering them susceptible to 5-FU. The majority of thyroid cancers expressed TP (14/19, 74%) suggesting that capecitabine would be converted into 5-FU. Consistent with previous findings in breast cancer, staining for TP was more common in thyroid cancers (14/19, 74%) than in normal thyroid (0/5, $P=0.006$ by Fisher's Exact Test) [3]. Unfortunately, a similar majority of thyroid cancers also expressed DPD (14/19, 74%) which might inactivate capecitabine and limit efficacy.

Overall, six tumors (6/19, 32%) had favorable expression profiles of TS, TP and DPD, suggesting that they would be sensitive to capecitabine therapy. All six were PTC. The average patient age, tumor size, and MACIS score for these PTC were similar to those of previous studies, suggesting that this group is fairly representative of PTC [22]. In contrast, 3/4 (75%) of the FTC and the only MTC in our study had unfavorable expression profiles suggesting that they might be resistant to capecitabine.

Using RT/PCR we detected TS and DPD in three cell lines derived from moderately differentiated PTC (NPA), poorly differentiated FTC (WRO) and anaplastic thyroid cancer (ARO) cells. TP was detected only in the PTC-derived NPA cells, suggesting that even moderately differentiated PTC might express sufficient TP to convert capecitabine into 5-FU. Unfortunately, DPD was also detected in the NPA cells.

In conclusion, 32% of all thyroid cancers in our study and almost half the PTC (43%) had favorable TP/DPD expression profiles. Additional study is warranted among older patients and those with more aggressive disease to determine if these findings can be generalized. However, the results do provide optimism for the potential use of capecitabine in the treatment of iodine-resistant PTC.

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