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TYROSINE KINASE EXPRESSION IS INCREASED IN PAPILLARY THYROID CARCINOMA OF CHILDREN AND YOUNG ADULTS

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1. ABSTRACT

Tyrosine kinases (TKs) are important candidate genes for malignant transformation and at least 21 different TKs have been identified in the thyroid gland. We hypothesized that the collective activity of these TKs might be increased in thyroid carcinoma and have association with the clinical behavior of individual tumors. To test this, we determined TK expression by immunohistochemistry in 74 archival thyroid tissue blocks (48 papillary thyroid carcinoma, PTC; 9 follicular thyroid carcinoma, FTC; 17 benign thyroid diseases) from children and young adults. Mean TK expression was greater for PTC (2.1 ± 0.11) than benign lesions (1.6 ± 0.2, $p = 0.027$), and also tended to be greater in FTC (2.1 ± 0.25, $p = 0.12$). Recurrence risk was three-fold greater for PTC with intense TK expression (4/15, 27%) than for PTC with minimal - moderate TK expression (3/33, 9.0%). However, this was not statistically significant ($p = 0.10$). In PTC, TK expression correlated with expression of the receptor for hepatocyte growth factor / scatter factor (cMET, $r = 0.31$, $p = 0.044$). In FTC, TK expression did not correlate with cMET, but tended to be greater in young patients ($r = -0.59$, $p = 0.09$). We conclude that TK expression is increased in PTC and possibly associated with an increased recurrence risk.

2. INTRODUCTION

The tyrosine kinases (TKs) are important candidates genes in the pathogenesis of malignant transformation (1-26). TK activity is intrinsic to membrane bound growth factor receptors, as well as cytosolic TKs. Amplification and / or over-expression of a number of TKs have been reported in human cancers (1, 5-11, 13-15, 17, 19, 21, 24, 25) including differentiated thyroid cancer in adults (27-60). In addition over-expression of TK activity has been directly related to the clinical outcome for carcinoma of the breast, ovary and colon, in which, over-expression of the TK (ERBB-2), is associated with a poor prognosis (5, 15, 24).

Several observations implicate TKs in the induction and control of differentiated thyroid cancer. Expression of the *ret*/PTC oncogenes (PTC-1, PTC-2, and PTC-3) has been detected in approximately 20% of adult papillary thyroid carcinoma (PTC) and almost 50% of PTC in children (28-30, 33-35, 37, 41-44, 47-49). These chimeric genes arise from chromosomal rearrangements which splice the tyrosine kinase domain of the *ret* proto-oncogene to different up-stream regulators. The rearrangements are found almost exclusively in PTC, result in over-expression of *ret* tyrosine kinase, and may be associated with a poor prognosis (42, 44, 47, 48).

A second important TK for the thyroid is cMET, a cell surface receptor which binds hepatocyte growth factor / scatter factor (HGF / SF). HGF/ SF is the single most potent stimulus for proliferation of the canine thyroid gland (50). Over-expression of cMET has been detected in PTC and is associated with an aggressive histological variant (tall-cell) and possibly an aggressive clinical course (27, 32, 51). Our own previous studies have shown that over-expression of cMET is associated with shortened recurrence-free survival for children and young adults with PTC.[52] Furthermore, increased co-expression of cMET and HGF/SF was even more highly correlated with the risk of recurrence for PTC (52).

Vascular endothelial growth factor (VEGF) is produced by the thyroid and binds two different receptors (FLT-1 and FLK-1) which both possess TK activity. The expression of VEGF is increased in goitrous disorders as well as thyroid cancer (53-55). We previously showed that the intensity of expression of VEGF and the type 1 VEGF tyrosine kinase receptor (FLT-1) are strongly correlated with the growth of PTC in children and young adults (56).

Additional TKs in the thyroid gland are regulated by the low molecular weight GTP binding protein, ras (57-58). Activating mutations of the *ras*

oncogene are common in adult PTC, and are thought to arise early during the malignant transformation into thyroid carcinoma. These *ras* mutations have specifically been shown to increase the expression of cMET as well (59).

A recent study identified at least 21 different TK's in the human thyroid gland (60). All of these TKs appear to be important in the regulation of cellular proliferation and any one or more might be important in malignant transformation. For this reason, we hypothesized that the collective activity of all TKs might be increased in thyroid carcinoma; and correlate with the biological behavior of individual cancers.

The present study was designed to determine TK activity in archival thyroid tissue blocks using an immunohistochemical method which specifically stained phosphorylated tyrosine residues. The results for benign and malignant tissues were compared, and the results for each tumor were correlated with the expression of cMET, VEGF, FLT-1, and FLK-1; the presence or absence of activating mutations in *ras* and *ret*/PTC; and the clinical outcome.

3. MATERIALS AND METHODS

3.1. Approval

This study received prior approval from the Human Use Committee of the Department of Clinical Investigation, Walter Reed Army Medical Center, Washington, DC. The study was funded by an intramural research grant from the Department of Clinical Investigation (WU 6414) Walter Reed Army Medical Center, Washington, DC.

3.2. Patients

The automated centralized tumor registry of the Department of Defense (ACTUR) was searched to identify all patients with differentiated thyroid carcinoma who were £ 21 years of age at the time of diagnosis. The clinical details of some patients in this group [137 patients with PTC and 33 with follicular thyroid cancer (FTC)] have been previously published (61). Records were used to construct a computerized data base which is maintained by the principal investigator and includes demographic features, tumor characteristics, surgical treatment, adjunctive therapy and clinical outcome. Additional tissue blocks and clinical histories were obtained from the Children's Hospital Medical Center, Cincinnati, OH. The extent of disease at diagnosis was classified according to the system of DeGroot *et al* (62). Class 1 disease was confined to the thyroid gland; Class 2 involved the regional lymph nodes; Class 3 either extended beyond the capsule or was inadequately resected; and Class 4 had distant metastasis. Recurrence was defined as the appearance of new disease (identified by radioactive iodine scan or biopsy) in any patient who had been free of disease (no disease palpable or identified by radioactive iodine scan) for a period of four months following initial therapy.

Archival tissue blocks were available for 48 patients with PTC, 9 patients with FTC, and a group of 17 benign thyroid lesions. The latter was comprised of 4 multinodular goiters, 3 Graves' disease, 3 follicular adenomas, 5 adenomatoid nodules, 1 lymphocytic thyroiditis, and 1 Hurtle cell adenoma. For analysis, the benign lesions were all grouped together and compared separately to PTC and FTC.

3.3. Immunohistochemistry

Sections from original, archival tissue blocks were sectioned and stained with hematoxylin and eosin to confirm the diagnosis. The sections immediately adjacent (5 µm) were used for immunohistochemistry. Sections were deparaffinized with xylene and rehydrated through a series of graded alcohol solutions followed by nuclease-free water. Sections were sequentially incubated with primary mouse monoclonal anti-TYR-PO4 antibody (1:200, RT, 1 hr, Oncogene Research, Cambridge, MA 02142); secondary biotinylated anti-mouse IgG; followed by the preformed avidin-biotinylated horseradish peroxidase complex; and diaminobenzidine chromogen (Unitect Mouse Immunohistochemistry System, Oncogene Research). Counterstaining was performed using Meyer's hematoxylin. A human melanoma tumor block was used as the positive control, and phosphate buffered saline was substituted for the primary antibody and used as the negative control. The intensity of staining was determined by two blinded, independent examiners and graded 1 (minimal to absent), 2 (moderate), and 3 (intense). The inter-observer agreement was 97%, and the few discordant slides were graded by a third examiner. The two scores in agreement were then used as the final intensity grade.

3.4. Data analysis and statistical comparisons

The intensity of staining for PTC, FTC and benign lesions were then compared and the intensity of staining for each of the cancers was correlated with the demographic features, histologic variant, focality of the tumor, size of the tumor, extent of disease at diagnosis (Class 1 - 4), and clinical outcome.

The intensity of TK immunostaining was also compared to that of cMET, VEGF, FLT-1, and FLK-1; as well as the presence of *ras* and *ret*/PTC mutations. Details of the methods and results for each of these have been previously published by our group using this same patient cohort (46, 49, 52, 58).

Statistical analysis was performed using SPSS for Windows 95 (Version 7.5, SPSS Inc., Chicago, IL). Correlations were performed using Pearson correlation, and recurrence-free survival was calculated using Kaplan-Meier survival curves with log-rank comparison. Non-parametric analysis was performed using either the Chi-square or Fisher's exact test as indicated.

4. RESULTS

The clinical features and intensity of TK staining for each patient with PTC are shown in table 1. The mean age was 16.0 ± 3.9 years (range 6 - 21 years); mean tumor size was 2.2 ± 1.5 cm (range 0.5 - 7.5 cm); and mean follow-up was for 5.5 ± 4.5 years (range 0 - 20.5 years). Twenty two patients (46%) had Class 1 disease; 19 (40%) had Class 2 disease; 5 (10%) had Class 3 disease; and 2

Table 1. Clinical Features And Expression Of TK In Patients With Papillary Thyroid Carcinoma (PTC)

CASE	AGE / SEX	SIZE (Cm)[1]	CLASS[2]	FOCALITY[3]	OPERATION[4]	^{131}IODINE ABLATION	RECURRENCE	TK GRADE
Class 1 - Recurrent								
1	21 / F	1.2	1	Multi	Total	Yes	Yes	1
2	14 / M	2	1	Uni	Total	Yes	Yes	3
Class 1 - No Recurrence								
3	14 / M	0.5	1	Multi	Total	No	No	3
4	19 / F	0.7	1	Uni	Subtotal	Yes	No	1
5	20 / F	0.7	1	Uni	Total	Yes	No	1
6**	19 / M	1.1	1	Uni	Subtotal	Unk	No	3
7	14 / F	1.2	1	Uni	Total	Unk	No	2
8	20 / M	1.2	1	Uni	Subtotal	No	No	1
9	21 / F	1.2	1	Multi	Total	Yes	No	1
10	16 / F	1.5	1	Multi	Total	Yes	No	2
11	18 / F	1.5	1	Uni	Total	Yes	No	1
12	11 / F	1.5	1	Uni	Subtotal	No	No	2
13	14 / F	1.6	1	Uni	Total	Unk	No	2
13	21 / F	1.8	1	Uni	Total	Unk	No	3
15	11 / F	2	1	Uni	Lobectomy	Unk	No	2
16	21 / F	2.3	1	Multi	Total	Unk	No	1
17	15 / F	2.4	1	Uni	Total	Yes	No	2
18	17 / F	2.4	1	Uni	Total	Yes	No	1
19	18 / F	2.4	1	Uni	Total	Unk	No	2
20	6 / F	2.6	1	Uni	Total	Unk	No	2
21	18 / F	2.9	1	Uni	Total	Yes	No	3
22	20 / M	5.0	1	Multi	Total	Yes	No	2
Class 2 - Recurrent								
23	14 / F	3	2	Multi	Total	Yes	Yes	3
24	13 / M	4.2	2	Multi	Subtotal	Yes	Yes	1
Class 2 - No Recurrence								
25	19 / M	0.5	2	Uni	Total	Yes	No	1
26	15 / F	0.7	2	Multi	Total	Yes	No	2
27	10 / F	0.8	2	Uni	Total	Yes	No	1
28	21 / F	1	2	Multi	Total	Unk	No	2
29	20 / F	1	2	Uni	Total	Yes	No	3
30	18 / F	1.8	2	Multi	Total	Yes	No	2
31	17 / F	2.0	2	Multi	Total	Yes	No	1
32	7 / F	2.0	2	Unk	Total	Unk	No	3
33	19 / F	2.3	2	Multi	Total	Yes	No	3
34	19 / F	2.5	2	Uni	Total	Unk	No	2
35	15 / F	2.5	2	Unk	Subtotal	Unk	No	3
36	16 / F	3.5	2	Unk	Total	Unk	No	3
37	13 / F	5.5	2	Multi	Subtotal	Unk	No	3
38	15 / F	7.5	2	Multi	Total	Yes	No	1
39	20 / F	Unk	2	Uni	Total	Yes	No	2
40	16 / F	Unk	2	Unk	Subtotal	Unk	No	2
41	14 / F	Unk	2	Multi	Total	Unk	No	2
Class 3 - Recurrent								
42	19 / F	2	3	Multi	Total	Yes	Yes	3
43	15 / M	NA	3	Unk	Total	Yes	Yes	2
Class 3 - No Recurrence								
44	17 / M	2.6	3	Multi	Total	Yes	No	1
45	10 / M	5	3	Uni	Subtotal	Yes	No	2
46	16 / M	Unk	3	Multi	Total	Unk	No	2
Class 4 - Recurrent								
47	6 / F	4.3	4	Multi	Near total	Yes	Yes	3
Class 4 - No Recurrence								
48	20 / F	0.5	4	Multi	Total	Yes	No	3

[1]Unk = unknown, [2]Class according to the classification system of DeGroot *et al* , [3] Focality indicates unifocal lesions (Uni) or multifocal lesions (Mutli) based on microscopic examination, [4] Operation refers to initial surgical procedure, ** Case 6 was exposed to radiation therapy 11 years before Papillary Thyroid Carcinoma (PTC).

Table 2. Clinical Features and Expression of TK in Patients with Follicular Thyroid Carcinoma (FTC)

CASE	AGE / SEX	SIZE (cm)	FOCALITY[1]	OPERATION[2]	^{131}IODINE ABLATION[3]	RECURRENCE	TK GRADE
Recurrent							
49	17 / F	2.2	Multi	Total	Yes	Yes	2
No Recurrence							
50	20 / F	0.2	Uni	Total	Yes	No	1
51	20 / M	1	Uni	Total	Yes	No	2
52	13 / M	1.2	Uni	Subtotal	Yes	No	3
53	17 / F	2.5	Uni	Total	Yes	No	2
54	19 / F	2.8	Uni	Total	Unk	No	3
55	16 / F	4	Uni	Total	Yes	No	2
56	21 / F	4	Uni	Subtotal	Yes	No	2
57	21 / F	4.5	Uni	Unk	Unk	No	1

[1] Focality indicates unifocal lesions (Uni) or multifocal lesions (Mutli) based on microscopic examination, [2] Operation refers to initial surgical procedure, [3] Unk = unknown

(4%) had Class 4 disease. Only 1 patient (case #6, 2.0 %) had received previous radiation exposure (11 years earlier for treatment of Hodgkin's lymphoma).

The clinical details and intensity of staining for each patient with FTC are shown in table 2. The mean age was 17.1 ± 4.4 years (range 7 - 21 years); mean tumor size was 2.3 ± 1.5 cm (range 0.2 - 4.5 cm); and mean follow-up was for 4.6 ± 3.5 years (range 1.5 - 14 years). The clinical characteristics of patients with PTC and FTC are similar to those of the larger series previously reported by our group (61).

The grading system for TK staining intensity is shown in figure 1. Slides are presented representing each of the TK staining grades (1 - 3). Figure 1(A) shows grade 1 staining. The patient presented with Class 2 disease and developed recurrent PTC after 13.3 years of follow-up. Figure 1(B) shows grade 2 staining. The patient presented with Class 1 disease and remained free of disease for the entire follow up interval (20 years). Figure 1(C) shows grade 3 staining. The patient presented with Class 4 disease and developed recurrent PTC 6 months following the initial treatment.

Figure 2 shows the results of TK staining for PTC compared to FTC and benign thyroid lesions. Mean staining intensity was greater for PTC (2.1 ± 0.11) compared to benign lesions (1.6 ± 0.2, p = 0.027). Overall, 71% of the PTC showed moderate - intense TK staining; whereas, the majority (70%) of benign lesions demonstrated only minimal - absent (grade 1) TK staining. Only 1 of the benign lesions revealed intense TK staining. Mean staining intensity for FTC (2.0 ± 0.25) was also greater than benign lesions but this difference did not achieve statistical significance (p = 0.12).

The intensity of TK staining for PTC was then analyzed with respect to the clinical outcome. There was a suggestion that TK expression might be increased in those with more extensive disease at diagnosis (mean intensity for class 1 = 1.9 ± 0.16, class 2 = 2.1 ± 0.19, class 3 = 2.0 ± 0.42, and class 4 = 3.0 ± 0, p = 0.23). However, the power of this observation was limited by the small number of patients with class 3 (n = 5) and class 4 (n = 2) disease.

Over time, seven patients developed recurrent disease. Of these, 4 (57%) had intense TK expression. In contrast, 41 patients did not develop recurrence; and of these, only 11 (27%) showed intense TK expression. Recurrent PTC were more likely to have intense TK expression (57% vs 27%) but this difference was not statistically significant (p = 0.11). Furthermore, 27% of the PTC with intense TK expression (4/15) developed recurrent disease. This was three-fold higher than the risk of recurrence found in the PTC with minimal or moderate TK expression (3/33, 9%); but this difference was not significant either (p = 0.10). There was also a suggestion that recurrence might develop earlier (r = 0.51, p = 0.24) for tumors with intense (25 ± 28 months, range 6 - 67 months, n = 4), or moderate TK expression (40 months, n = 1) compared to those with minimal TK expression (85 ± 103 months, 12 - 159, n=2). There was no correlation between TK expression and tumor size, nor was there any difference in TK expression between male and female patients or between unifocal and multifocal tumors.

The relationship between TK expression and outcome for FTC was then examined. Figure 3 shows the relationship between TK expression and patient age. There appeared to be a decline in TK activity with increasing age, but this only approached statistical significance (r = -0.59, p = 0.09). Over time, one patient with FTC developed recurrent disease. TK expression in this FTC was similar to the mean TK expression found in FTC which did not recur (2 vs 2.0 ± 0.23). Just as for PTC, there was no correlation between TK expression and patient sex, tumor size, or tumor focality.

Within the group of PTC, the relationships between overall TK expression and the expression of several individual tyrosine kinases which have been previously published for these same patients, were then examined (46, 49, 52, 58). Figure 4 shows the relationship between the expression of TK and cMET. There was a modest, but significant correlation (r = 0.31, p =0.044) between the overall expression of TK and that of cMET. Overall TK expression was surprisingly lower in PTC which contained mutations in the *ras* oncogene (1.0 ± 0 vs 2.2 ± 0.14 in PTC without *ras* mutations, p = 0.027).

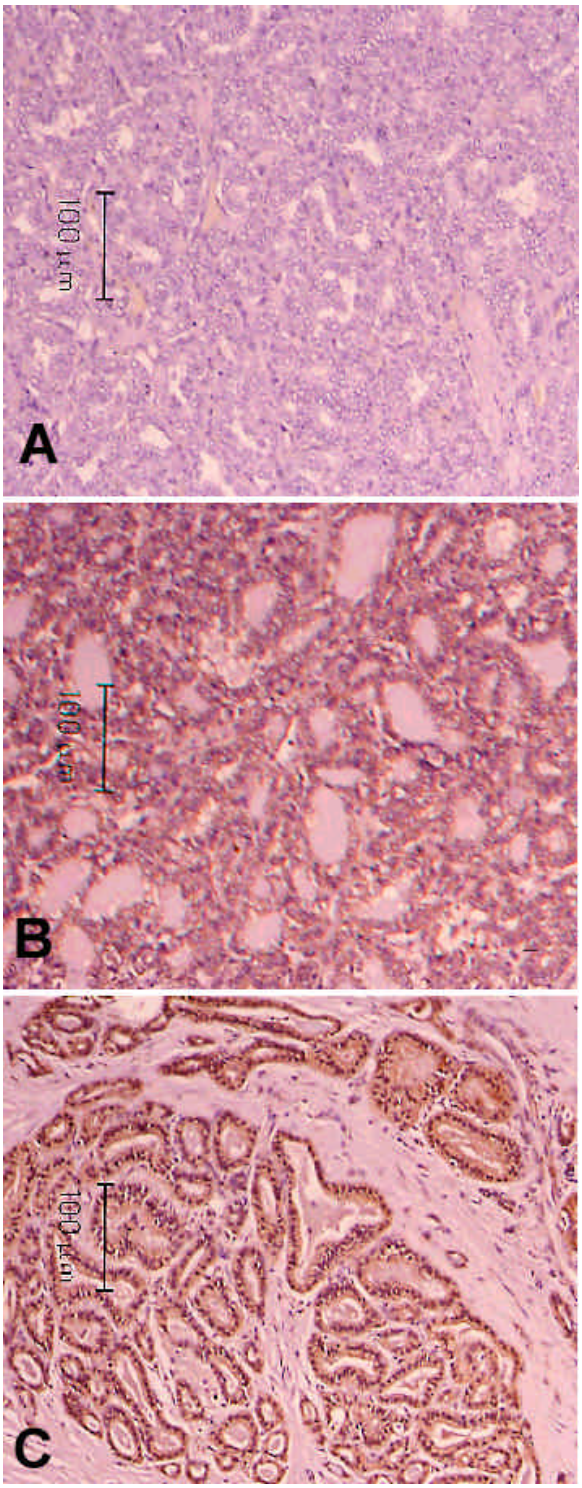

Figure 1. Representative examples of each grade of TK staining intensity. Three PTC were stained for expression of tyrosine-phosphate and graded as follows: Figure 1(a) Grade 1 = absent - minimal; Figure 1(b) Grade 2 = moderate; and Figure 1(c) Grade 3 = intense. Sections are representative only and were not obtained from the same patient. All sections are shown at 100 x magnification.

In addition, overall TK activity tended to be lower (1.8 ± 0.2 vs 2.4 ± 0.2, p = 0.07) in PTC containing *ret* oncogene rearrangements (PTC-1, PTC-2, or PTC-3) when compared to PTC without *ret*/PTC mutations. Overall TK expression tended to correlate (r = 0.30, p = 0.07, data not shown) with expression of the type 2 VEGF receptor (FLK-1); but not with VEGF (r = 0.02, p = 0.89), or the type 1 VEGF receptor (FLT-1, r = 0.08, p = 0.65).

5. DISCUSSION

Several observations implicate TKs in the induction and control of differentiated thyroid cancer (27-60). The *ret*/PTC oncogenes (PTC-1, PTC-2, and PTC-3) are present in approximately 20% of adults and 50% of children with PTC; result in over expression of *ret* TK activity; and might be associated with a poor prognosis (28-30, 33-35, 37, 41-44, 47, 48). Over-expression of the HGF/SF receptor (cMET) has been associated with an aggressive histological variant and an aggressive clinical course in adults with PTC (42, 44, 47, 48). Our own studies have shown that increased expression of cMET is associated with a high recurrence risk for PTC in children and young adults (52); while increased expression of VEGF and the type 1 VEGF receptor (FLT-1) are associated with increased size of PTC (58). These observations suggest that the TK receptors and their cognate ligands may have important roles in the control of differentiated thyroid cancer.

To our knowledge, this is the first study to examine the relationships between overall TK activity and the clinical outcome for individual patients or the expression of individual TKs in the thyroid. The results of the current study support the importance of tyrosine kinases in defining the clinical and biological behavior of PTC and FTC in children and young adults.

The data show that TK expression is increased in PTC compared to benign thyroid lesions in children and young adults (mean intensity 2.1 ± 0.11 vs 1.6 ± 0.2, p = 0.027). Furthermore, there were suggestions that recurrent PTC were more likely to exhibit intense TK expression (p = 0.11) and that PTC with intense TK expression might have an increased risk of recurrence (p = 0.10). There was also a suggestion that TK expression might be greater in PTC with more extensive disease at diagnosis (p = 0.12), however, the power of these latter observations was limited by the small number of patients with recurrent or extensive disease.

TK expression in FTC also tended to be greater than benign lesions (2.1 ± 0.25 vs 1.6 ± 0.2, p = 0.12) and to decline with increasing age (r = - 0.59, p = 0.09). However, neither of these differences reached statistical significance, perhaps because of the smaller number of FTC available for our study. There was no correlation for either PTC or FTC between TK expression and tumor size; nor was there any difference in TK expression between male and female patients or between unifocal and multifocal tumors.

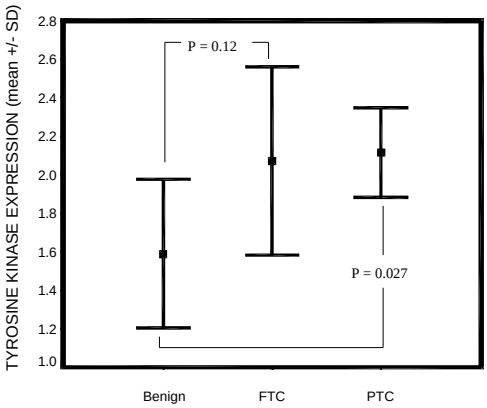

Figure 2. The expression of TK is compared for PTC, FTC, and benign thyroid lesions. TK expression was greater in PTC than benign lesions (p = 0.027) and tended to be greater in FTC as well (p = 0.12).

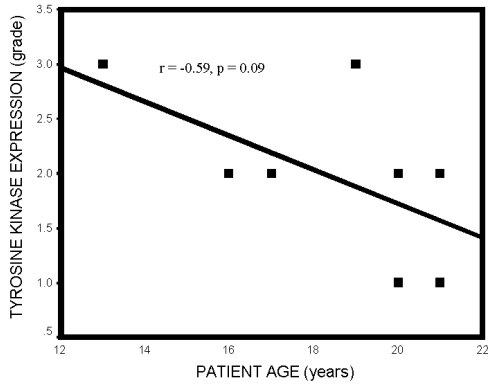

Figure 3. TK expression as related to the age of patients with FTC. TK expression tended to decline with increasing patient age (r = - 0.59, p = 0.09) for the patients with FTC.

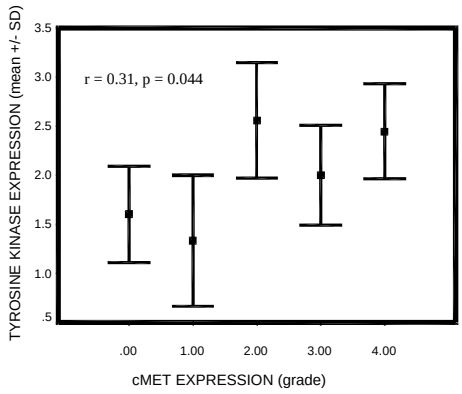

Figure 4. Relationship between TK and cMET expression for PTC. TK expression was compared to the expression of cMET. Data presented are mean TK expression for each grade of cMET. More intense TK and cMET expression were found in the same sections (r = 0.31, p = 0.044).

The data do provide independent validation for the importance of cMET in PTC. Overall TK expression was related to the specific expression of the HGF/SF receptor (cMET, r = 0.31, p = 0.044), and possibly the type 2 VEGF receptor (FLK-1, r = 0.30, p = 0.07). Among the tumors for which both TK and cMET data were available, 12 PTC showed intense TK expression. Of these, 7 (58%) also showed moderate - intense expression of cMET. These findings suggest that cMET contributes a significant portion of the overall TK activity expressed by PTC. However, there were five PTC with intense TK expression (5/12, 42%), which showed only minimal or absent cMET expression. This suggests the additional possibility that there could be other TK enzymes which contribute to the overall TK activity found in PTC. The identity of these TK enzymes is not clear from our data.

Surprisingly, activating mutations in the *ras* oncogene, and rearrangements resulting in *ret*/PTC-1, PTC-2, or PTC-3 were associated with reduced TK activity (1.0 ± 0 vs 2.2 ± 0.14, p = 0.027; and 1.8 ± 0.2 vs 2.4 ± 0.2, p = 0.07; respectively). These findings are contrary to the results we anticipated since *ras* and *ret* have both been implicated in the up-regulation of cMET expression (59). Our data offer no explanation for this finding. However, the data do suggest that overall TK activity may be determined by some other, quantitatively more significant, TK enzymes. In support of this theory, Tanaka *et al* (60) previously identified 21 different TK enzymes in the thyroid. Of these, the insulin like growth factor-1 receptor, platelet derived growth factor receptor, TrkE, Axl and epidermal growth factor receptor were most abundant. It is possible, that overall TK activity might correlate with the expression of these abundant TK enzymes. We are currently developing techniques to examine this possibility.

In conclusion, our data have shown that TK expression is increased in malignant thyroid tumors compared to benign thyroid lesions and may be associated with a greater risk of recurrence. Our data also suggest an interesting relationship between TK expression and the age of patients with FTC. TK expression tended to be greater in FTC from the youngest patients. This last observation suggests the intriguing possibility that TK activity could be developmentally regulated in the thyroid.

6. ACKNOWLEDGMENT

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Uniformed Services University of the Health Sciences, the Department of the Army, or the Department of Defense.

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