ORIGINAL ARTICLE

Expression of c-Kit, Flk-1, and Flk-2 Receptors in Benign and Malignant Tumors of Follicular Epithelial Origin

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Background: Vascular endothelial growth factor (VEGF) is a key regulator of physiologic as well as pathologic angiogenesis. The response of VEGF to endothelial cell mitogenesis and survival, as well as angiogenesis and microvascular permeability, is mainly mediated through its receptor-2, VEGFR2 (kinase domain receptor or fetal liver kinase-1, KDR or Flk-1). This study aimed to detect the expression of VEGFR2 in various forms of thyroid tumors. In addition, the expression of Flk-2 (receptor for Flt-3) and c-Kit (receptor for steel locus factor), which shows strong similarity to Flk-1, was also examined in thyroid tumors.

Methods: RT-PCR analyses of c-Kit and immunohistochemical staining of c-Kit, Flk-1, and Flk-2 were performed in archived samples of 18 papillary thyroid carcinoma (PTC), 9 follicular thyroid carcinoma (FTC), 12 follicular adenoma (FA), and 7 nodular goiter (NG) samples. The data were correlated to clinicopathologic features.

Results: By RT-PCR analyses, c-Kit expression was detected in 22% (4/18) of PTC, 22% (2/9) of FTC, 25% (3/12) of FA, and 57% (4/7) of NG samples. However, positive immunostaining signals of c-Kit were only observed in 17% (3/18) of PTC samples, and not in the others. Similarly, Flk-1 expression was only detected by immunohistochemistry in 67% (12/18) of PTC and 43% (3/7) of NG samples, and not in the others. Interestingly, the expression of Flk-2 was found in 89% (16/18) of PTC, 89% (8/9) of FTC, 75% (9/12) of FA, and 29% (2/7) of NG samples. An inverse relationship of thyroid cancer size with Flk-2 expression was found.

Conclusion: Flk-2 expression was detected in various forms of thyroid tumors and increased Flk-2 expression was correlated with thyroid tumors with increased transforming activity, suggesting that Flk-2 is involved in pathogenic development of thyroid malignancy. Similarly, Flk-1 expression was also found in some thyroid tumors, while the expression of c-Kit-mediated pathways may not play a major role in thyroid tumorigenesis. [*J Chin Med Assoc* 2006; 69(2):74–79]

Key Words: c-Kit, Flk-1, Flk-2, thyroid tumors

Introduction

Angiogenesis is an absolute requirement in normal tissues for embryogenesis, skeletal growth, and reproductive functions. It is also associated with several pathologic conditions such as tumor growth and its metastases. The process of branching out of new blood vessels from preexisting vasculature is essential for thyroid organogenesis.[1] It has also been found in the thyroid in the disease process of goiter, Graves disease, thyroiditis, and neoplastic transformation.[2]

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Vascular endothelial growth factor (VEGF) is a key regulator of physiologic as well as pathologic angiogenesis. The response of VEGF is mediated through its receptor-1 and -2, VEGFR1 (Flt-1 or Fms-related tyrosine kinase 1) and VEGFR2 (KDR or Flk-1). In general, VEGFR2 is the major mediator of endothelial cell mitogenesis and survival, as well as angiogenesis and microvascular permeability, whereas VEGFR1 acts as a negative regulator to sequester VEGF and prevents its interaction with VEGFR2. The action of VEGF and its receptors has been implicated in the growth and metastasis of thyroid tumors.[3–8] Increased expression of VEGF, VEGFc, KDR/Flk-1, and Tek was found in human thyroid tumor and higher VEGF expression was correlated with tumor size in adults.[6] Among them, VEGFc and VEGFR3 mRNA were found in benign thyroid tumors as well as thyroid carcinoma,[4] and the expression of VEGFc may contribute to intrathyroid spread of tumor cells.[3,5] Overexpression of tyrosine kinase Flt and c-Met was associated with increased recurrence of thyroid cancer.[3] The expression of VEGF was found to be higher in differentiated thyroid cancer tissue.[7] Moreover, Katoh et al[8] found significantly increased expression of VEGF in undifferentiated carcinoma cells near necrotic foci where the supply of oxygen was reduced. By immunohistochemistry, Klein et al[9] also found higher expression of VEGF in metastatic thyroid tumors than in nonmetastatic tumors. The involvement of VEGF in angiogenesis and growth of thyroid tumors was further supported by the observation that the inhibition of VEGF secretion by phenylacetate[10] or VEGF antisense oligonucleotides[11] was correlated with growth inhibition of thyroid cancer cells. These results together suggest that angiogenesis plays an important role in the growth and metastasis of thyroid cancer. Although angiogenesis is a prerequisite in the pathogenic development of thyroid tumors, many molecules that have been implicated as positive regulators of angiogenesis were found to be weakly or negatively expressed in subsets of thyroid tumors. These angiogenic factors include VEGF, VEGFc, VEGFR3, KDR/Flk-1 and Tek,[6] hepatocyte growth factor,[12] and metalloproteinase-9.[13] To explore other factors that may be associated with angiogenesis and metastasis of thyroid tumors, this study aimed to examine the expression of c-Kit (CD117), Flk-1 (VEGFR2 or KDR), and Flk-2 (Flt-3, STK1, or CD135), and to determine whether they work cooperatively to promote vascular formation in thyroid tumors.

Methods

Patients

Thyroid tumor and adjacent tissues were obtained from patients who underwent thyroid surgery at the Taipei Veterans General Hospital. Informed consent was obtained from each patient. All the carcinoma patients underwent total thyroidectomy, while others had lobectomy or subtotal thyroidectomy. The operation was performed by a single surgeon and the specimens were snap frozen immediately after resection and stored in liquid nitrogen until use. Normal thyroid specimens were obtained from the grossly normal part of the thyroid and were also histologically verified. The patient group consisted of 18 histologically confirmed papillary thyroid carcinomas (PTCs), 9 follicular thyroid carcinomas (FTCs), 12 follicular adenomas (FAs), and 7 nodular goiters (NGs). Among them, 5 were males and 41 were females with age ranging from 15 to 81 years. The cancer group (PTC and FTC) versus benign tumor group (FA and NG) were compared by Chi-square test or independent t-test. Differences were considered statistically significant when the p value was less than 0.05.

Immunohistochemistry

Sections 5 μm thick were prepared from the archived wax blocks of thyroid tumor and their nontumor counterpart tissues, and mounted on silane-coated slides and dried at 56°C for 2 hours. After dewaxing, sections were pretreated by microwave at 800 W for 20 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval. The sections were then incubated with polyclonal antibodies against c-Kit (dilution 1:50; MBL, Japan), Flk-1 (dilution 1:100; Santa Cruz Biotechnology, CA, USA), and Flk-2 (dilution 1:100; Santa Cruz Biotechnology), respectively, at 4°C for 16 hours. In a sequential order, the tissue slides were incubated with a nonspecific blocker (10% nonimmune rabbit serum; Zymed, San Francisco, CA, USA) for 10 minutes, biotinylated with universal immunoglobulin G (IgG) for 10 minutes, streptavidin peroxidase conjugate for 10 minutes, AEC substrate chromogen for 5 minutes, and then counterstained with hematoxylin for 5 minutes. Pre-immune rabbit IgG was used as the negative control. Slides were reviewed based on tumor cells in 5 random × 20 microscopic fields by the same pathologist (A.H.Y.). The stains were graded according to the percentage of cancer cells showing positive signals: "+" represents a positive finding in less than 25% of tumor cells; "++" represents a positive finding in 25–50% of tumor cells; "+++" represents a positive finding in 50–75% of tumor cells; "++++" represents a positive

finding in more than 75% of tumor cells; "–/+" represents a faint stain between a positive and negative finding of tumor cells.

Isolation of RNA and RT-PCR

Total RNA was isolated from 50 to 100 mg of frozen tissues in 1 mL of TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation. One microgram of total RNA was converted to cDNA by MMLV-reverse transcriptase (Roche, Penzberg, Germany) in 20 μL of reaction mixture containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L DTT, and 1 mmol/L dNTP with 0.625 μg random primers following the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out in a 10-μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.01% (w/v) gelatin, 2 mmol/L dNTP, 0.1 μg/mL of each primer, 1 μL of properly diluted cDNA, and 2.5 units *Taq* DNA polymerase, respectively. Forty cycles of PCR (94°C for 15 seconds, 53°C for 30 seconds, and 72°C for 40 seconds) were performed with primers specific for c-Kit (sense primer, 5'-GCT GCC AAG TCT CTG TGA ATA-3' and antisense primer 5'-ACC ACA GTC CAT GCC ATC AC-3' ; GenBank accession no. BC004558) transcripts, and 25 cycles of PCR (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute) were performed with primers specific for *GAPDH* (sense primer, 5'-TCC ACC ACC CTG TTG CTG TA-3' and antisense primer, 5' -GGC ACG GTT GAA TGT AAG GCT-3' ; GenBank accession no. NM-002046) transcripts. PCR products were separated by agarose gel electrophoresis and examined under UV after ethidium bromide staining.

Results

Using immunohistochemistry, we examined the expression of c-Kit, Flk-1, and Flk-2 in thyroid tumors from 46 patients. Table 1 shows the clinical information of the 46 patients and the relation to the expression of

Table 1. Clinical information for the 46 patients and the relation to the expression of c-Kit, Flk-1, and Flk-2

Diagnosis	Total n = 46	PTC n = 18	FTC n = 9	FA n = 12	NG n = 7	p value (PTC+FTC vs FA+NG)
Male/Female	5/41	0/18	0/9	4/8	1/6	0.311*
Age, yr, mean ± SD (range)	42.7 ± 16.0 (15–81)	45.4 ± 15.2 (25–81)	36.4 ± 15.2 (23–71)	41.8 ± 15.5 (15–63)	45.3 ± 21.0 (21–72)	0.932†
Flk-1						
–	31	6	9	12	4	
–/+	10	7	0	0	3	
+	5	5	0	0	0	0.067*
Flk-2						
–	11	2	1	3	5	
–/+	6	1	1	3	1	
+	19	6	6	6	1	
++	8	7	1	0	0	
+++	2	2	0	0	0	0.005*
c-Kit						
–	43	15	9	12	7	
–/+	3	3	0	0	0	0.257*
Tumor size, cm	n = 27 2.8 ± 1.6	2.4 ± 1.4	3.6 ± 1.7			

** PTC + FTC versus FA + NG compared by Chi-square test. †PTC + FTC versus FA + NG compared by independent t-test. $p < 0.05$ is considered statistically significant. PTC = papillary thyroid carcinoma; FTC = follicular thyroid carcinoma; FA = follicular adenoma; NG = nodular goiter; n = case number.*

c-Kit, Flk-1, and Flk-2. Figure 1 shows the positive immunohistochemical staining of Flk-2 in PTC. Intense cytoplasmic staining was observed in tumor cells. Similarly, positive cytoplasmic staining was observed in tumor cells of FTC (Figure 2). Among the 46 samples examined, positive signals of Flk-2 expression were found in 89% (16/18) of PTC, 89% (8/9) of FTC, 75% (9/12) of FA, and 29% (2/7) of NG samples. Figure 3 summarizes the profile of Flk-2 expression in various forms of thyroid tumors. The Flk-2 expression showed a trend of inverse relationship to thyroid cancer size with a p value of 0.182 (Figure 4). The expression of c-Kit was examined with both immunostaining and RT-PCR analyses, while the expressions of Flk-1 and Flk-2 were detected by immunostaining alone because it had better expression. By RT-PCR analysis, we detected c-Kit expression in 22% (4/18) of PTC, 22% (2/9) of FTC, 25% (3/12) of FA, and 57% (4/7) of NG samples. However, positive immunocytostaining signals of c-Kit were only observed in 17% (3/18) of PTC samples, and not in the others. Similarly, Flk-1 expression was only detected by immunohistochemistry in 67% (12/18) of PTC and 43% (3/7) of NG samples, and not in the others.

Discussion

In the current study, we examined the expression of c-Kit, Flk-1, and Flk-2 in human thyroid tumors. c-Kit and Flk-2/Flt-3 are cytokine tyrosine kinase receptors that are expressed and function in early human hematopoiesis. Through its ability to promote *ex vivo*

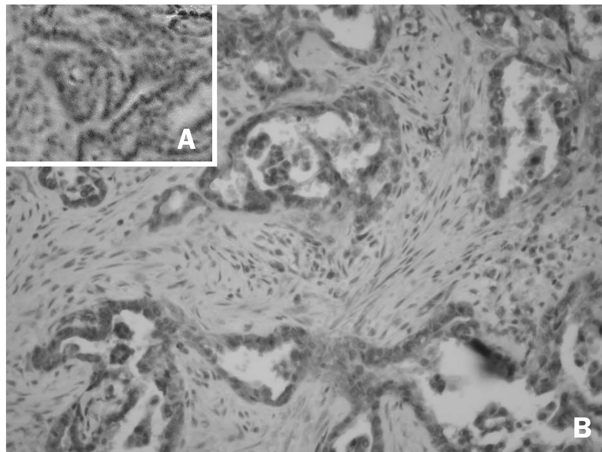

Figure 1. Papillary thyroid carcinoma with control (A) and positive expression of Flk-2 (B).

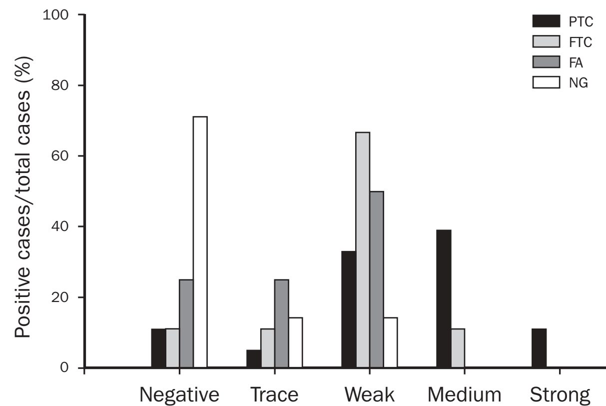

Figure 3. Flk-2 expression in thyroid tumors. PTC = papillary thyroid carcinoma (n = 18); FTC = follicular thyroid carcinoma (n = 9); FA = follicular adenoma (n = 12); NG = nodular goiter (n = 7).

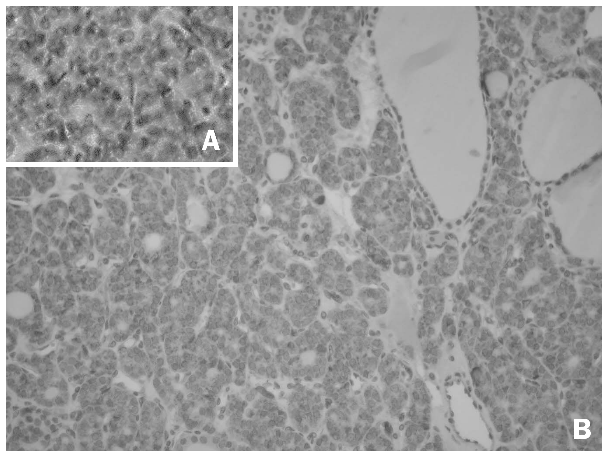

Figure 2. Follicular thyroid carcinoma with control (A) and positive expression of Flk-2 (B).

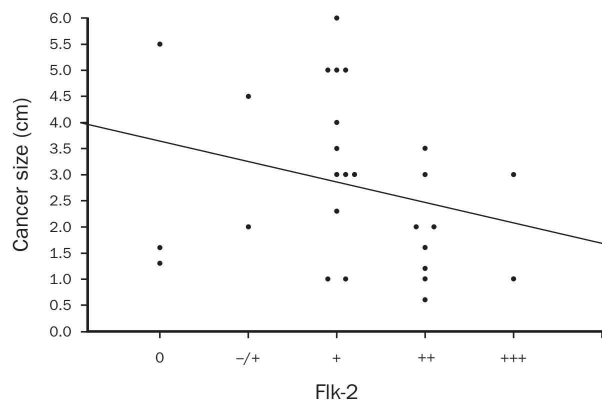

Figure 4. The relationship between the differentiated thyroid cancer sizes (18 of papillary cancer plus 9 of follicular cancer) and its Flk-2 expression.

expansion and onco-retroviral transduction of human hematopoietic progenitors, the Flk-2/Flt-3 ligand has emerged as a key stimulator of human hematopoietic stem cells,[14] which are critical for angiogenesis.[15] Flk-1, also known as VEGFR2, is also essential for the development of hematopoietic stem cells in the embryo. Although c-Kit, Flk-1, and Flk-2 are important factors involved in both physiologic and pathologic angiogenesis, only Flk-1 has been shown to express in human thyroid cancers.[6] Using immunohistochemistry, we have shown in this study that all 3 were expressed in human thyroid cancers.

Flk-2/Flt-3 is a marker in hematopoietic stem cell differentiation.[16] Aberrant expression and activating mutations of Flk-2 have been described and linked to poor prognosis in acute myeloid leukemia, although it can be inhibited by bis(1H-2-indoolyl)-1-methanones.[17] Zeigler et al[18] have shown that stem cells expressing Flk-2 have a lower percentage of cells that remain at G_0 as compared with the cells that do not express Flk-2, suggesting that Flk-2 may promote cells into active cycling status. In this study, Flk-2 is most frequently expressed in thyroid tumors, as compared with Flk-1 and c-Kit. The expression of Flk-2 was widely detected in both PTC and FTC, and to a lesser degree, in FA and NG samples ($p = 0.005$) (Table 1). These findings suggest that Flk-2 is involved in the pathogenic development of thyroid cancers. How the Flk-2 ligand/Flk-2 signal axis contributes to thyroid tumorigenesis merits further investigation. Our study also evidenced the expression of Flk-2 expressed in most growing thyroid tumors with stronger expression in cancer lesions (Figure 3). We also showed that Flk-2 expression in thyroid tumors has a trend of inverse relationship to cancer size (Figure 4). It remains to be determined why this result is different from the report of Bounone et al[6] in 1999 and whether the early rapidly growing thyroid tumors need more angiogenic activity or more activated hematopoietic stem cells. This study also demonstrated that a higher percentage of FTC expressed Flk-2 as compared with FA (89% vs 75%). This has always been a dilemma in the differential diagnosis of FA and FTC. Other than using the parameters related to the expression of metalloproteinase-9,[13] nuclear morphometry, and quantitation of angiogenesis,[19] our Flk-2 expressions still seem difficult to serve as a marker to differentiate FTC from FA.

c-Kit is a proto-oncogene that expresses mainly in hematopoietic stem cells and lymphocyte progenitor cells. c-Kit expression has also been described in a number of cancers, including uterine carcinosarcoma and neuroblastomas;[20,21] c-Kit expression is associated with neuroblastomas with a favorable prognosis.[20,21] By RT-PCR analysis, we showed that 22–57% of thyroid tumors of various types expressed c-Kit to different degrees. Immunohistochemistry also confirmed the expression of c-Kit in PTC. It is not clear whether the absence of c-Kit expression in other types of thyroid tumors by immunohistochemistry is due to a minute amount of c-Kit protein expression that is beyond detection. Nevertheless, both RT-PCR and immunohistochemistry showed that 17–22% of PTC expressed c-Kit. It warrants further study to evaluate the relation of c-Kit expression to the prognosis of PTC in a larger panel of patients. In parallel experiments, we found positive Flk-1/KDR expression in 67% (12/18) of PTC and trace signals in 43% (3/7) of NG samples, and none of the FA and FTC samples showed any signals. Flk-1/KDR encodes VEGFR2, which has been implicated in the positive regulation of VEGF-mediated responses. These results suggested that the VEGF-mediated signaling pathway plays an important role in the development of NG and PTC, but it may play a less important role in the pathogenesis of FA and FTC.

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