# Diagnostic Value of 16 Cellular Tumor Markers for Metastatic Thyroid Cancer: An Immunohistochemical Study

HUASHENG LIANG[1], YUHUA ZHONG[1], ZUOJIE LUO[2], YU HUANG[3], HUADE LIN[4], SONG ZHAN[5], KAIQING XIE[6] and QINGDI QUENTIN LI[7]

[1]*Beihai Institute of Endocrine and Metabolic Diseases, and* [5]*Department of Pathology, Ninth Affiliated Hospital of Guangxi Medical University, Beihai 536000, China;*
[2]*Department of Endocrinology, First Hospital of Guangxi Medical University, Nanning 530021, China;*
[3]*Department of Hepatobiliary and Endocrine Surgery, Guangxi Provincial Hospital, Nanning 530021, China;*
[4]*Department of Hepatobiliary and Endocrine Surgery, Pingnan People's Hospital, Pingnan 537300, China;*
[6]*Nanfang Hospital, Southern Medical University, Guangzhou 510515, China;*
[7]*National Institutes of Health, Bethesda, MD 20892, U.S.A.*

**Abstract.** *Background: The prognosis for thyroid cancer differs between metastatic and non-metastatic cases. To identify biomarkers useful for thyroid cancer diagnosis and to establish a marker panel for the early detection of metastatic thyroid carcinoma, this study compared histomorphological features and biomarker expression profiles in thyroid carcinomas according to pathological diagnoses. Patients and Methods: Thyroid carcinoma samples were obtained from 113 consecutive patients who underwent resection at multiple centers between 2001 and 2008. These cases included 63 metastatic thyroid tumors (34 papillary carcinomas, 20 follicular carcinomas, 9 undifferentiated carcinomas) and 50 non-metastatic thyroid tumors (36 papillary carcinomas, 14 follicular carcinomas). Tissue microarrays constructed using the 113 samples were analyzed by immunohistochemistry for the expression of 16 protein markers: MMP9, VEGF-C, E-cadherin, MMP2, PPARγ, PCNA, CXCR4, PTEN, C-myc, PTTG, HBME-1, p16, p53, FHIT, bFGF and hTERT. The clinicopathological variables with diagnostic significance were determined by multivariate analysis, and the predictive values of the identified biomarkers for metastasis in thyroid carcinoma were determined by receiver operating characteristic (ROC) curve analysis. Results: The expression of six proteins, VEGF-C, MMP2, CXCR4, PTTG, HBME-1 and bFGF, was up-regulated in metastatic compared to non-metastatic thyroid carcinoma. Multiple factor binary ordinal logistic regression analysis showed that MMP2, PTTG, VEGF-C, CXCR4 and bFGF were independent factors associated with the metastatic status of thyroid carcinoma. ROC curve analysis of these five proteins revealed that VEGF-C and bFGF were the most useful protein markers for the diagnosis of metastatic thyroid cancer. Conclusion: MMP2, PTTG, VEGF-C, CXCR4 and bFGF are potential cellular tumor markers for identifying thyroid cancer with greater risk for metastasis and the novel combination of VEGF-C and bFGF as biomarkers may improve the accuracy of early detection and the differential diagnosis between metastatic and non-metastatic thyroid carcinoma.*

*Correspondence to:* Prof. Yuhua Zhong, Ninth Affiliated Hospital of Guangxi Medical University, Beihai 536000, China. E-mail: zhongyh111@163.com, or Dr. Huasheng Liang, Beihai Institute of Endocrine and Metabolic Diseases, Beihai 536000, China. E-mail: flowchaos@yahoo.com.cn

*Key Words:* Thyroid cancer, biomarker, diagnosis, sensitivity, specificity, metastasis, immunohistochemistry.

Thyroid carcinoma, the most common endocrine-related cancer, is a heterogeneous group of tumors that tend to metastasize, but fail to exhibit sufficient characteristics to achieve classification as a specific histological type. Matrix metalloproteinase (MMP) 2, MMP9, vascular endothelial growth factor (VEGF)-C, E-cadherin, peroxisome proliferator-activated receptor gamma (PPARγ), proliferating cell nuclear antigen (PCNA), chemokine receptor CXCR4, phosphatase and tensin homolog (PTEN), C-myc, pituitary tumor-transforming gene (PTTG), Hector Battifora mesothelial-1 (HBME-1), p16, p53, fragile histidine triad (FHIT), basic fibroblast growth factor (bFGF) and human telomerase reverse transcriptase (hTERT) have been studied as tumor markers for the detection, diagnosis and monitoring of different types of carcinomas. Some biomarkers such as MMP2, VEGF-C, HBME-1 and bFGF have been proposed for assessing the risk for metastasis of thyroid cancer in individuals (1-6). However, the intense search for predictive

molecular biomarkers of metastasis has not yet translated into clinical use.

In the current study, thyroid carcinoma tissues from 113 patients were immunohistochemically examined. The aims of the study were to: characterize the protein expression profiles of 16 biomarkers in thyroid carcinomas with and without metastasis; identify cellular tumor biomarkers useful for the early detection of thyroid carcinoma and establish a marker panel for the differential diagnosis between metastatic and non-metastatic thyroid carcinomas.

## Patients and Methods

*Patient recruitment, inclusion and exclusion criteria*. Patients were identified consecutively treated for thyroid carcinomas that were histologically confirmed by the pathology departments of five hospitals, which included the Ninth Affiliated Hospital of Guangxi Medical University, First Affiliated Hospital of Guangxi Medical University, Guangxi Provincial Hospital, Guangxi Pingnan People's Hospital, and Nanfang Hospital of Southern Medical University in China between 2001 and 2008. All the patients recruited at the hospitals provided signed written and informed consent. All the patients had undergone surgical resection of primary thyroid gland disease and had histopathological slides available for review. Paraffin-embedded tissue blocks were also available for all the patients. Patients with non-thyroid tumors, benign tumors or metastases to the thyroid gland and those having had only fine-needle aspiration or biopsy of the thyroid were excluded. All the available clinical, pathological, treatment and follow-up data were reviewed and updated for the 113 patients who had undergone thyroidectomy for thyroid carcinoma. The study protocol was approved by the institutional review board of Guangxi Medical University School of Medicine.

*Treatment of thyroid cancer patients*. All the patients had undergone primary treatment according to the standard of care at the Ninth Affiliated Hospital of Guangxi Medical University, China. This included thyroidectomy with or without concomitant solid organ (lymph node, pancreas, spleen, kidney, bone or liver) resection as necessary to achieve complete removal of all grossly evident disease. Adjuvant treatment in the form of radiotherapy or chemotherapy had been administered as part of the standard care or as part of a clinical trial.

*Pathological review*. All the available operative reports and information contained in the institutional pathology database and all the pathology reports of the primary nodules were reviewed to confirm the completeness of resection. In addition, all the available autopsy reports were reviewed. A mean of five hematoxylin and eosin-stained slides per patient was reviewed in conjunction with the corresponding pathological records, but without knowledge of the clinical data. All 113 histologically confirmed thyroid carcinomas in this study were included in the analysis. According to the WHO classification of follicular thyroid, papillary thyroid and undifferentiated thyroid carcinomas, there were 70 papillary thyroid carcinomas (34 metastatic, 36 non-metastatic); 34 follicular thyroid carcinomas (20 metastatic, 14 non-metastatic) and nine undifferentiated thyroid carcinomas (all metastatic). The thyroid lesions were divided into two groups, metastatic (63 cases) and non-metastatic (50 cases).

*Clinical and pathological variables*. The clinical and pathological data included patient age, gender and nodule size, and the expression levels of the 16 potential biomarkers: MMP9, VEGF-C, E-cadherin, MMP2, PPARγ, PCNA, CXCR4, PTEN, C-myc, PTTG, HBME-1, p16, p53, FHIT, bFGF and hTERT.

*Tumor samples*. Tissue microarrays (TMAs) were constructed using cores (1 mm in diameter) taken from appropriate areas of the formalin-fixed paraffin-embedded tissue blocks of all 113 thyroid carcinomas. The tissue cores were arrayed in a recipient paraffin block, using a tissue arraying instrument (Beecher Instruments, Silver Spring, MD, USA). Serial sections of 4-μm thickness were cut from the TMA blocks and mounted on glass slides. The use of TMAs to immunohistochemically assess the expression of PTTG, E-cadherin, VEGF-C, MMP9, MMP2, CXCR4 and bFGF in whole sections of nodules was validated in a study of thyroid glands with benign *versus* cancerous nodules.

*Immunohistochemical staining*. The thyroid tissue samples were reviewed to confirm diagnosis and construction, and tissues that had previously been shown by immunohistochemical analysis to express the antigens of interest were used as positive controls in the TMA sections. Normal tissues were used as baseline controls. The TMA sections were deparaffinized, rehydrated in a graded series of alcohol and processed using the avidin-biotin immunoperoxidase method. The sections were placed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave oven for 15 min. This procedure was followed by staining with most of the antibodies used in this study; for staining with a few antibodies, the sections were incubated in preheated 0.05 M Tris-HCl (pH 7.6) containing 0.05% trypsin and 0.05% $CaCl_2$ for 5 min at 37˚C before the microwave treatment (Table I). After antigen retrieval, the sections were incubated in 10% normal bovine serum for 30 min, followed by incubation with appropriately diluted primary antibody overnight at 4˚C, or at another temperature as indicated (Table I). Diaminobenzidine was used as a chromogen and hematoxylin was used as a nuclear counterstain. The rate of lost cases attributable to tissue damage ranged from 1 to 10% for the different protein markers.

*Cancer classification*. A pathological image analysis system (DMR-Q550; Leica, Solms, Germany) was used to independently review and score the slides with respect to the intensity of staining and percentage of tumor cells stained. All the stained sections were examined at high magnification. The percentage of immunoreactivity was determined as continuous data, from undetectable (0) to homogeneously stained (100%), for each marker. The staining intensity for each marker was graded as: 0, achromatic; 1, light yellow; 2, light brown and 3, dark brown. We then estimated the percentage of immunopositive cells for each marker and assigned the following scores: 0, <5% of cells; 1, 5-25% of cells; 2, 26-50% of cells; 3, 51-75% of cells and 4, >75% of cells. Finally, the staining intensity score was multiplied by the immunopositive cell percentage score for each marker and these values were classified as: 0, negative (–); 1-4, weakly positive (+); 5-8, moderately positive (++) and 9-12, strongly positive (+++) as reported previously (6).

*Statistical data analysis*. All the data were analyzed statistically using SPSS ver. 13 software (SPSS, Inc., Chicago, IL, USA). The significance level was considered to be $\alpha$=0.05. The measurement

Table I. *Details of antibodies and experimental conditions used for immunohistochemistry.*

| Biomarker | Antibody clone | Supplier | Dilution | Incubation conditions |
|---|---|---|---|---|
| PTTG | Polyclonal | Zhongshan Golden Bridge Bio, Beijing, China | 1:50 | 1 h, 37˚C |
| CXCR4 | Polyclonal | Abcam, Cambridge, UK | 1:75 | 1 h, 37˚C |
| MMP9 | 56-2A4 | Maixin-Bio, Fuzhou, China | Dispensed | 1 h, 37˚C |
| VEGF-C | Polyclonal | R&D, Minneapolis, MN, USA | 1:75 | Overnight, 37˚C |
| bFGF | Polyclonal | Santa Cruz, Santa Cruz, CA, USA | 1:150 | 75 min, 37˚C |
| p53 | 2Q375 | Santa Cruz | 1:50 | 1 h, 37˚C |
| PPARγ | E-8 | Santa Cruz | 1:50 | Overnight, 4˚C |
| HBME-1 | HBME-1 | Abcam | 1:75 | 1 h, 37˚C |
| MMP2 | MMP2/8B4 | Abcam | 1:75 | 1 h, 37˚C |
| C-myc | Polyclonal | Abcam | 1:100 | 1 h, 37˚C |
| p16 | Polyclonal | Maixin-Bio | Dispensed | 1.5 h, 37˚C |
| FHIT | Polyclonal | Zhongshan Golden Bridge Bio | 1:50 | 1 h, 37˚C |
| hTERT | Y182 | Epitomics, Burlingame, CA, USA | 1:50 | Overnight, 4˚C |
| PCNA | F-2 | Santa Cruz | 1:50 | 1.5 h, 37˚C |
| PTEN | Polyclonal | Santa Cruz | 1:75 | 1 h, 37˚C |
| E-cadherin | Polyclonal | Abcam | 1:50 | 1 h, 37˚C |

Table II. *Protein expression profiles of thyroid carcinomas (TCs) with and without metastasis.*

| Protein marker | Non-metastatic TC (n=50) | | | | Metastatic TC (n=63) | | | | *P** |
|---|---|---|---|---|---|---|---|---|---|
| | Immunoreactivity | | | | Immunoreactivity | | | | |
| | – | + | ++ | +++ | – | + | ++ | +++ | |
| MMP9 | 9 | 8 | 12 | 21 | 10 | 11 | 16 | 26 | 0.987 |
| VEGF-C | 26 | 9 | 10 | 5 | 4 | 25 | 21 | 13 | 0.000 |
| E-cadherin | 31 | 14 | 5 | 0 | 45 | 17 | 1 | 0 | 0.129 |
| MMP2 | 3 | 11 | 28 | 8 | 1 | 6 | 33 | 23 | 0.033 |
| PPARγ | 21 | 19 | 7 | 3 | 28 | 21 | 12 | 2 | 0.769 |
| PCNA | 12 | 20 | 9 | 9 | 12 | 18 | 16 | 17 | 0.381 |
| CXCR4 | 15 | 18 | 14 | 3 | 10 | 15 | 24 | 14 | 0.022 |
| PTEN | 22 | 17 | 10 | 1 | 19 | 23 | 18 | 3 | 0.400 |
| C-myc | 6 | 14 | 17 | 13 | 11 | 15 | 23 | 14 | 0.811 |
| PTTG | 27 | 12 | 10 | 1 | 12 | 18 | 23 | 10 | 0.000 |
| HBME-1 | 0 | 9 | 12 | 29 | 11 | 13 | 16 | 23 | 0.009 |
| p16 | 25 | 16 | 8 | 1 | 21 | 19 | 17 | 6 | 0.112 |
| p53 | 10 | 17 | 15 | 8 | 17 | 18 | 18 | 10 | 0.836 |
| FHIT | 26 | 15 | 9 | 0 | 31 | 14 | 12 | 0 | 0.140 |
| bFGF | 20 | 21 | 9 | 0 | 9 | 17 | 30 | 7 | 0.000 |
| hTERT | 8 | 16 | 12 | 14 | 12 | 15 | 19 | 17 | 0.747 |

Data are reported as number of patients. Immunoreactivity score: –, negative; +, weakly positive; + +, positive; + + +, strongly positive. *$P$-values<0.05 represent significant differences in the percentage of immunopositive (+ + and + + +) patients between the metastatic and non-metastatic groups.

data were analyzed by analysis of variance or a rank sum test and paired comparisons. The categorical data were analyzed using the Chi-square test. The significant factors were determined by binary logistic regression. Receiver operating characteristic (ROC) curve analysis of the sensitivity and specificity was used to calculate the "best value" of each variable for predicting thyroid tumor status and to select the variables with the best values. Successive partitioning of ROC curves was used to compare predictive accuracy and the best cut-off point for the selected variables.

## Results

*Biomarker expression in thyroid carcinomas*. The data for the expression of the 16 potential biomarkers (MMP9, VEGF-C, E-cadherin, MMP2, PPARγ, PCNA, CXCR4, PTEN, C-myc, PTTG, HBME-1, p16, p53, FHIT, bFGF and hTERT) in the metastatic and non-metastatic thyroid carcinoma groups are shown in Table II. Out of the 16 biomarkers assessed, PTTG,

CXCR4, VEGF-C, MMP2 and bFGF showed significantly ($p<0.05$) different expression between the two groups (Table II). The down-regulation of E-cadherin showed a tendency to be associated with metastasis.

*Factors related to metastatic status*. To study the relationships between the tumor characteristics and the clinical parameters, pathological and clinic factors were analyzed using binary logistic regression. The thyroid carcinoma category, as ordinal multiple data, was the dependent variable and the clinical and laboratory parameters were independent variables (Table III). Eleven factors were used for binary ordinal logistic regression analysis with forward stepwise factor selection. The significance level was set at $\alpha=0.05$, and the elimination level was set at $\alpha=0.10$.

In multiple binary logistic regression analysis, only PTTG, MMP2, VEGF-C, CXCR4 and bFGF retained significant independent associations with the metastatic status of thyroid carcinoma, with a positive correlation between the expression levels of these five proteins and metastatic thyroid carcinoma (Table IV). The results of the goodness-of-fit test (Chi-square, 11.864; degrees of freedom, 7; $p=0.105$) indicated that the observed proportion of patients with tumors without metastasis was similar to the predicted proportion in the derivation group. The calibration curves for the derivation data demonstrated good calibration of the prediction rule.

*Predictive values of marker expression for metastasis*. The predictive values of the five variables that reached the level of statistical significance in the multivariate analysis (PTTG, MMP2, VEGF-C, CXCR4 and bFGF) were examined by ROC curve analysis (Table V). A threshold value that yielded an appropriate tradeoff between sensitivity and specificity (*i.e.*, probability of a tumor without metastasis) was adopted. The area under the ROC curve was largest for VEGF-C and bFGF, making these the most useful markers (Figure 1). The cut-off values for VEGF-C and bFGF were 0.5 and 1.5, respectively.

To better understand the utility of VEGF-C and bFGF applied individually and in combination as biomarkers for diagnostic screening, their sensitivity for the detection of metastatic *versus* non-metastatic thyroid carcinoma was examined. Table VI shows the sensitivity and specificity of VEGF-C and bFGF in serial and parallel tests, with cut-off threshold values of VEGF-C – negative, + to +++ positive and bFGF – to + negative, ++ to +++ positive.

Table III. *Quantification of variables for multiple factor binary logistic regression analysis*.

| Variable | Target | Quantification |
|---|---|---|
| Y | Item | Non-metastatic=0, Metastatic=1 |
| X1 | Tumor size | $cm^3$ |
| X2 | Gender | Female=1, Male=0 |
| X3 | Age (years) | 0-30=1, 30.01-60=2, >60=3 |
| X4 | VEGF-C | –, 0; +, 1; ++, 2; +++, 3 |
| X5 | MMP2 | –, 0; +, 1; ++, 2; +++, 3 |
| X6 | CXCR4 | –, 0; +, 1; ++, 2; +++, 3 |
| X7 | PTTG | –, 0; +, 1; ++, 2; +++, 3 |
| X8 | HBME-1 | –, 0; +, 1; ++, 2; +++, 3 |
| X9 | bFGF | –, 0; +, 1; ++, 2; +++, 3 |
| X10 | Type of carcinoma | Papillary carcinoma=1<br>Follicular carcinoma=2<br>Undiferentiated carcinoma=3 |

## Discussion

Although some biomarkers have been used to differentiate metastatic and non-metastatic thyroid carcinoma (4, 7-14), the role of biomarker panels in the diagnosis of metastatic thyroid carcinoma is not fully understood. In this study, we have demonstrated that up-regulated levels of PTTG, HBME-1, MMP2, CXCR4, VEGF-C and bFGF and a down-regulated level of E-cadherin tended to be associated with metastasis in thyroid carcinoma patients. Among these, VEGF-C and bFGF appeared to be the most useful markers for differentiating metastatic and non-metastatic thyroid carcinomas. These potential protein markers identified by immunohistochemical analysis must be verified by other methods and using larger numbers of representative tumor materials.

Out of the markers studied, VEGF-C was the most strongly correlated with the metastatic status of thyroid cancer. VEGF-C was originally found to be associated with metastasis in a study using a human prostatic adenocarcinoma cell line, where it was shown to stimulate lymphatic proliferation, and VEGF-C has been shown to be expressed in carcinomas that had metastasized to lymph nodes. Liang and colleagues (11) were the first to study VEGF-C expression in thyroid tumors, and Bunone *et al*. (15) published a study on the expression of VEGF-C in papillary thyroid carcinomas. Yu and coworkers (16) also examined VEGF-C expression in thyroid carcinoma. The present results were consistent with earlier studies (11, 15, 16) in which metastatic thyroid tumors were found to be immunohistochemically positive for VEGF-C, whereas lesions without metastases were negative or minimally positive for VEGF-C.

In the present study, the immunohistochemical staining of E-cadherin did not show a significant difference between metastatic and non-metastatic thyroid cancer, which did not agree with previously reported results describing an association between the down-regulation of E-cadherin and metastasis in thyroid carcinoma (17-19). This disparity

Table IV. *Multiple factor binary logistic regression analysis of protein biomarker expression.*

| Factor | Coefficient of regression | Standard error | Wald statistic | *P*-Value | Exp(B) | 95% CI for Exp(B) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper |
| VEGF-C | 0.917 | 0.299 | 9.393 | 0.002 | 2.501 | 1.392 | 4.496 |
| MMP2 | 1.203 | 0.400 | 9.044 | 0.003 | 3.332 | 1.521 | 7.299 |
| CXCR4 | 1.028 | 0.321 | 10.283 | 0.001 | 2.796 | 1.492 | 5.242 |
| PTTG | 1.016 | 0.318 | 10.226 | 0.001 | 2.761 | 1.482 | 5.145 |
| bFGF | 1.402 | 0.382 | 13.450 | 0.000 | 4.062 | 1.920 | 8.591 |
| Constant | –7.398 | 1.492 | 24.581 | 0.000 | 0.001 | | |

95% CI, 95% confidence interval.

suggests that E-cadherin may not be useful for identifying metastatic thyroid carcinoma.

Matrix metalloproteinases participate in the degeneration of extracellular matrix and are associated with carcinogenesis. In the present study, similarly high expression levels of MMP2 and MMP9 were associated with metastasis-positive thyroid carcinoma, suggesting that the expression of MMP2 and MMP9 may persist in the metastatic stages of thyroid cancer. Many studies have shown a significantly positive association between MMP-2 immunostaining and thyroid carcinoma, both metastatic and non-metastatic. Furthermore, derivative metastases have frequently shown strong MMP9 expression (4, 6). Tan and colleagues (20) recently reported increased MMP2 expression in tumor tissue with extrathyroidal metastasis. In contrast, Cavalheiro *et al*. (21) found no correlation between MMP2 expression and the presence of nodal metastases, although similar findings have not been widely reported. One emerging opinion is that dynamic, irreversible modulation of MMPs occurs during the progression of thyroid carcinoma.

The formation of new blood vessels from pre-existing vasculature is critical in the development, growth, and metastasis of carcinomas. The search for potential stimuli of angiogenesis has yielded numerous candidates, including PTTG and bFGF. PTTG has been shown to promote angiogenesis, a key rate-limiting step in tumor progression, by the up-regulation of bFGF and vascular endothelial growth factor (VEGF). Boelaert *et al*. (10) found that PTTG and bFGF were overexpressed in thyroid carcinomas. Increased bFGF mRNA expression was independently associated with lymph node invasion and distant metastasis in a study of 27 differentiated thyroid carcinomas. By contrast, the current study confirmed statistically significant differences in the expression of PTTG and bFGF between metastatic and non-metastatic lesions in thyroid carcinoma, suggesting that they may be useful biomarkers for the differential diagnosis between metastatic and non-metastatic thyroid carcinomas.

Table V. *Cut-off values for protein biomarker expression in thyroid carcinomas with and without metastasis.*

| Biomarker | Cut-off value | ROC curve area | 95% CI for Exp(B) | |
|---|---|---|---|---|
| | | | Lower | Upper |
| MMP2 | 0.5 | 0.646 | 0.544 | 0.748 |
| CXCR4 | 1.5 | 0.658 | 0.558 | 0.759 |
| PTTG | 0.5 | 0.718 | 0.624 | 0.813 |
| bFGF | 1.5 | 0.738 | 0.646 | 0.829 |
| VEGF-C | 0.5 | 0.721 | 0.622 | 0.820 |

95% CI, 95% confidence interval.

Metastasis to regional lymph nodes is a common step in cancer progression. Recent evidence has suggested that the production of CXCR4 by tumors promotes lymph node metastasis. CXCR4 is expressed by a variety of solid tumors, including papillary thyroid carcinoma (22-24). The present observations confirmed strong expression of CXCR4 in 23 out of 27 of the cases of papillary metastatic carcinomas and a significant difference in CXCR4 expression between the metastatic and non-metastatic thyroid carcinomas, in agreement with other reports (3).

A number of factors, including age, gender, tumor size and protein marker expression levels, have been used to assess the risk for metastatic cancer. In the current study, only VEGF-C, PTTG, MMP2, CXCR4 and bFGF retained independent value as markers of metastasis in the multivariate analysis. The effectiveness of linear regression for the identification of optimal markers to distinguish metastatic from non-metastatic tumors in the present study suggests that this may be applicable as a general method for optimizing antibody panels to distinguish between other histologically similar conditions. The quality of a marker is defined by the extent to which its sensitivity and specificity remain high at the threshold set to define marker positivity. As shown in the present results, the ROC curve is a

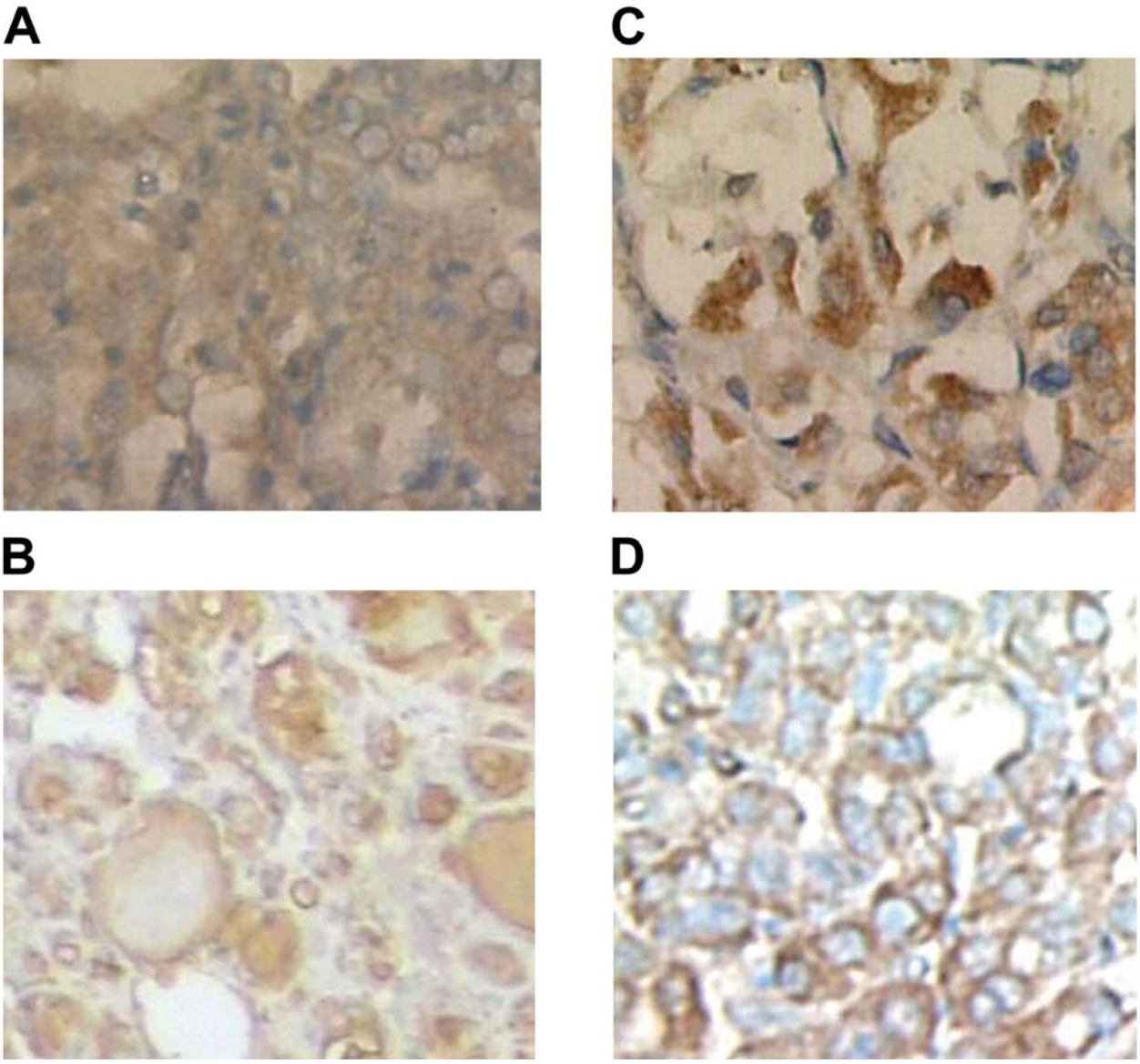


Figure 1. *Immunohistochemical staining of bFGF and VEGF-C in papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) tissues. Tissue sections (4-μm thickness) were cut from formalin-fixed, paraffin-embedded human PTC and FTC tissue specimens. Immunohistochemical staining showing strong immunoreactivity of bFGF protein to anti-bFGF antibody in PTC with metastasis (A) and FTC with metastasis (B) and of VEGF-C protein to anti-VEGF-C antibody in PTC with metastasis (C) and FTC with metastasis (D). Representative tissue sections are shown (original magnification, ×200).*

Table VI. *Predictive values of protein biomarkers for clinical diagnosis of thyroid carcinomas with and without metastasis.*

| Factor | Accuracy (%) | Sensitivity (%) | Specificity (%) | Positive predictive value (%) | Negative predictive value (%) | kappa |
|---|---|---|---|---|---|---|
| VEGF-C | 75.2 | 52.0 | 93.7 | 86.7 | 71.1 | 0.476 |
| bFGF | 69.0 | 82.0 | 58.7 | 61.2 | 80.4 | 0.393 |
| Serial test* | 70.8 | 90.0 | 55.6 | 61.6 | 87.5 | 0.435 |
| Parallel test** | 73.5 | 44.0 | 96.8 | 91.7 | 68.5 | 0.431 |

*In a serial test, a result is positive only when both VEGF-C and bFGF are positive. **In a parallel test, a result is positive when either VEGF-C or bFGF is positive.

convenient measurement for comparing the overall quality of markers. VEGF-C, with the highest sensitivity with relatively high specificity, was clearly the best marker for positively identifying metastatic thyroid cancer; bFGF achieved greater than 71.9% sensitivity for identifying metastasis, with a specificity of about 69.2%. Although MMP2, CXCR4 and PTTG were also capable of identifying metastasis with high sensitivity, the larger areas under the ROC curves for VEGF-C and bFGF make them optimal markers.

The combined use of VEGF-C and bFGF could improve their utility in screening and diagnosing metastatic thyroid carcinoma. The positive predictive value of a multimodal screening program may be increased by incorporating these biomarkers to detect and subsequently differentiate metastatic from non-metastatic thyroid cancer. Based on the present data, serial testing with fixed thresholds for VEGF-C and bFGF would provide 80% specificity and 71.1% sensitivity for discerning patients with metastatic thyroid carcinoma from those with non-metastatic thyroid carcinoma (Table VI).

In summary, the present immunohistochemical study of the expression of potential protein markers in thyroid cancer and the correlations between their expression levels and the metastatic status of thyroid cancer demonstrates the up-regulation of MMP2, PTTG, VEGF-C, CXCR4 and bFGF protein expression in 63 subjects diagnosed with and treated for metastatic thyroid cancer. Moreover, the combination of VEGF-C and bFGF was the best predictor of the risk for metastasis. A validated set of these protein biomarkers may be useful for the accurate identification and prognostic monitoring of thyroid cancer patients who exhibit metastasis. The use of this novel biomarker combination of VEGF-C and bFGF could improve the accurate diagnosis of patients with thyroid carcinoma and provide a screening method for the early detection of metastatic thyroid cancer. Further characterization and validation of the clinical significance of the identified potential biomarkers in a larger number of thyroid carcinomas with and without metastasis are required.

## Acknowledgements

We are grateful to Xiujiu Liang for her technical assistance and to Su Xuan for helping with the manuscript preparation. This study was supported by grants from the Applied Basic Research Programs of Science and Technology Commission Foundation of Guangxi Province (No. 0339080) and Guangxi Provincial Science Foundation for Young Scientists (No. 0728108).

## References

1 Daa T, Kodama M, Kashima K, Yokoyama S, Nakayama I and Noguchi S: Identification of basic fibroblast growth factor in papillary carcinoma of the thyroid. Acta Pathol Jpn *43*: 582-589, 1993.

2 Yasuoka H, Kodama R, Hirokawa M, Takamura Y, Miyauchi A, Sanke T and Nakamura Y: CXCR4 expression in papillary thyroid carcinoma: induction by nitric oxide and correlation with lymph node metastasis. BMC Cancer *8*: 274-280, 2008.

3 Wagner PL, Moo TA, Arora N, Liu YF, Zarnegar R, Scognamiglio T and Fahey TJ: The chemokine receptors CXCR4 and CCR7 are associated with tumor size and pathologic indicators of tumor aggressiveness in papillary thyroid carcinoma. Ann Surg Oncol *15*: 2833-2841, 2008.

4 Maeta H, Ohgi S and Terada T: Protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitors of metalloproteinase 1 and 2 in papillary thyroid carcinomas. Virchows Arch *438*: 121-128, 2001.

5 Friguglietti CU, Mello ES, Castro IV, Filho GB and Alves VA: Metalloproteinase-9 immunoexpression and angiogenesis in thyroid follicular neoplasms: relation to clinical and histopathologic features. Head Neck *22*: 373-379, 2000.

6 Maruyama S, Kawata R and Shimada T: Study of matrix metalloproteinase-2 and -9 in thyroid papillary cancer. Nippon Jibiinkoka Gakkai Kaiho *103*: 499-505, 2000.

7 Batistatou A, Charalabopoulos K, Nakanishi Y, Vagianos C, Hirohashi S, Agnantis NJ and Scopa CD: Differential expression of dysadherin in papillary thyroid carcinoma and microcarcinoma: correlation with E-cadherin. Endocr Pathol *19*: 197-202, 2008.

8 Coppee F, Depoortere F, Bartek J, Ledent C, Parmentier M and Dumont JE: Differential patterns of cell cycle regulatory proteins expression in transgenic models of thyroid tumours. Oncogene *17*: 631-641, 1998.

9 Laco J and Ryska A: The use of immunohistochemistry in the differential diagnosis of thyroid gland tumors with follicular growth pattern. Cesk Patol *42*: 120-124, 2006.

10 Boelaert K, McCabe CJ, Tannahill LA, Gittoes NJ, Holder RL, Watkinson JC, Bradwell AR, Sheppard MC and Franklyn JA: Pituitary tumor transforming gene and fibroblast growth factor-2 expression: potential prognostic indicators in differentiated thyroid cancer. J Clin Endocrinol Metab *88*: 2341-2347, 2003.

11 Liang QC, Wei QY and Fan SQ: Expression of VEGF-C and angiogenesis, and lymphangiogenesis in papillary thyroid carcinoma. Zhong Nan Da Xue Xue Bao Yi Xue Ban *31*: 414-416, 2006.

12 Siironen P, Ristimäki A, Narko K, Nordling S, Louhimo J, Andersson S, Haapiainen R and Haglund C: VEGF-C and COX-2 expression in papillary thyroid cancer. Endocr Relat Cancer *13*: 465-473, 2006.

13 Franchi A, Massi D, Palomba A, Biancalani M and Santucci M: CDX-2, cytokeratin 7 and cytokeratin 20 immunohistochemical expression in the differential diagnosis of primary adenocarcinomas of the sinonasal tract. Virchows Arch *445*: 63-67, 2004.

14 Ordonez NG: The diagnostic utility of immunohistochemistry in distinguishing between mesothelioma and renal cell carcinoma: a comparative study. Hum Pathol *35*: 697-710, 2004.

15 Bunone G, Vigneri P, Mariani L, Butó S, Collini P, Pilotti S, Pierotti MA and Bongarzone I: Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am J Pathol *155*: 1967-1976, 1999.

16 Yu XM, Lo CY, Chan WF, Lam KY, Leung P and Luk JM: Increased expression of vascular endothelial growth factor C in papillary thyroid carcinoma correlates with cervical lymph node metastases. Clin Cancer Res *11*: 8063-8069, 2005.

17 Kato N, Tsuchiya T, Tamura G and Motoyama T: E-cadherin expression in follicular carcinoma of the thyroid. Pathol Int *52*: 13-18, 2002.
18 Brecelj E, Frkovic Grazio S, Auersperg M and Bracko M: Prognostic value of E-cadherin expression in thyroid follicular carcinoma. Eur J Surg Oncol *31*: 544-548, 2005.
19 Mitselou A, Ioachim E, Peschos D, Charalabopoulos K, Michael M, Agnantis NJ and Vougiouklakis T: E-cadherin adhesion molecule and syndecan-1 expression in various thyroid pathologies. Exp Oncol *29*: 54-60, 2007.
20 Tan H, Ye K and Wang Z: Clinicopathologic evaluation of immunohistochemical CD147 and MMP-2 expression in differentiated thyroid carcinoma. Jpn J Clin Oncol *38*: 528-533, 2008.
21 Cavalheiro BG, Junqueira CR and Brandao LG: Expression of matrix metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinase 2 (TIMP-2) in medullary thyroid carcinoma: prognostic implications. Thyroid *18*: 865-871, 2008.
22 Tallini G, Garcia-Rostan G, Herrero A, Zelterman D, Viale G, Bosari S and Carcangiu ML: Downregulation of p27kip1 and CXCR4/Mib1 labeling index support the classification of thyroid carcinoma into prognostically relevant categories. Am J Surg Pathol *23*: 678-685, 1999.
23 Hwang JH, Hwang JH, Chung HK, Kim DW, Hwang ES, Suh JM, Kim H, You KH, Kwon OY, Ro HK, Jo DY and Shong M: CXC chemokine receptor 4 expression and function in human anaplastic thyroid cancer cells. J Clin Endocrinol Metab *88*: 408-416, 2003.
24 De Falco V, Guarino V, Avilla E, Castellone MD, Salerno P, Salvatore G, Faviana P, Basolo F, Santoro M and Melillo RM: Biological role and potential therapeutic targeting of the chemokine receptor CXCR4 in undifferentiated thyroid cancer. Cancer Res *67*: 11821-11829, 2007.

*Received May 23, 2011*
*Revised July 19, 2011*
*Accepted July 20, 2011*