Increased Expression of FGF19 Contributes to Tumor Progression and Cell Motility of Human Thyroid Cancer

Xiliang Zhang, MD1, Zhonghua Wang, MD1, Lei Tian, MS1, Jiangping Xie, MS1, Guijun Zou, MS1, and Futing Jiang, MD1

Abstract

Objective. Numerous reports indicate a role for aberrant expression of fibroblast growth factor 19 (FGF19) in tumor development and progression, and several drugs have been developed to target it. The aim of this study was to investigate the clinical significance of FGF19 and examine whether it plays any roles in progression of thyroid cancer.

Study Design. Translation research.

Setting. Navy General Hospital of Chinese PLA, China.

Subjects and Methods. Expression patterns of FGF19 protein in 100 paired formalin-fixed and paraffin-embedded cancerous and adjacent noncancerous tissues from patients with thyroid cancer were detected by immunohistochemistry. Then, in vitro migration and invasion assays of siRNA-targeted FGF19-transfected cells were performed.

Results. Positive immunostaining of FGF19 protein expression was localized in cytoplasm with or without membrane of malignant cells and was observed in 82 (82.0%) of 100 patients with thyroid cancer. Statistically, the expression level of FGF19 protein in thyroid cancer tissues was significantly higher than that in normal tissues. In addition, FGF19 overexpression was significantly associated with the advanced tumor node metastasis staging (P = .008), the presence of extrathyroidal invasion (P = .01), lymph nodes metastasis (P = .01), and distant metastasis (P = .02). Furthermore, knockdown of FGF19 by transfection of siRNA-FGF19 could efficiently suppress the migration and invasion abilities of thyroid cancer cells in vitro.

Conclusion. Our data revealed that the increased expression of FGF19 might be involved in the malignant behaviors of thyroid cancer, highlighting its potential as a molecular marker for early diagnosis and as a possible target for therapeutic intervention of this disease.

Keywords

fibroblast growth factor 19, thyroid cancer, motility, immunohistochemistry, small interfering RNA

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Thyroid cancer, the most common endocrine malignancy, has posed a major challenge to oncologists due to the rapid increase in its incidence.1 In the United States, approximately 33,000 new cases are diagnosed as thyroid cancer annually, and >1500 patients die of this cancer each year.2 Based on the differentiation degree, thyroid cancers are divided into (1) well-differentiated thyroid cancers, including papillary thyroid cancer and follicular thyroid cancer, which account for >90% of all thyroid cancers, and (2) poorly differentiated thyroid cancer or anaplastic thyroid cancer, the latter of which is completely undifferentiated, very aggressive, and always fatal.3 In recent years, there has been a better understanding of the molecular mechanisms, as well as rapid advancements in molecular diagnostic technology, which all contribute to the recognition on thyroid carcinogenesis and allow significant progress in the study of diagnostic, prognostic, and therapeutic options for patients with thyroid cancer.4 However, growing evidence shows that tumor cells in about 10% of patients with well-differentiated thyroid cancers may lose the ability to uptake radioiodine or become poorly differentiated or dedifferentiated, which may result in the recurrence of disease and death.5 Therefore, it is of great clinical significance to investigate other molecular events during the progression of thyroid cancer to identify optimal biomarkers for early-stage diagnosis, prognosis, and innovative therapy.

Fibroblast growth factor 19 (FGF19)—a member of FGFs that constitute a family of 22 structurally related polypeptides with diverse biological activities—is located at chromosomal region 11q13, and it can function as an endocrine factor that plays a crucial role in regulating various cellular processes, such as glucose, lipid, and vitamin D
metabolisms, as well as bile acid synthesis. Numerous reports indicate a role for aberrant expression of FGF19 in tumor development and progression under pathologic states, and several drugs have been developed to target it. Functionally, it may be involved in the cellular differentiation, growth, cell cycle, and motility of malignant cells. Recent studies have reported the overexpression of FGF19 mRNA and/or protein in various human cancers. For example, Miura et al found that FGF19 was significantly overexpressed in hepatocellular carcinoma tissues as compared with the corresponding noncancerous liver tissues. Similarly, Hyeon et al indicated that FGF19 overexpression was correlated with the early recurrence of patients with hepatocellular carcinoma but not with the late recurrence. Buhmeida et al indicated that the expression level of FGF19 protein was dramatically increased in invasive ductal carcinoma of breast and correlated with worse prognosis. Nagamatsu et al also reported that FGF19 overexpression might be associated with biochemical recurrence after radical prostatectomy by promoting cell proliferation and epithelial-mesenchymal transition of prostate cancer. Collectively, these findings implied that FGF19 could promote aggressive tumor progression of many cancer types.

However, the biological role of FGF19 in thyroid cancer remains an enigma. To investigate the clinical significance of FGF19 and to examine whether it plays any roles in progression of thyroid cancer, we performed immunohistochemistry to detect the expression patterns of FGF19 protein in 100 paired formalin-fixed and paraffin-embedded cancerous and adjacent noncancerous tissues obtained from patients with thyroid cancer. Then, the associations between FGF19 expression and clinicopathologic characteristics of patients with thyroid cancer were statistically evaluated. After that, in vitro migration and invasion assays of siRNA-targeted FGF19-transfected cells were performed.

**Materials and Methods**

**Patients and Tissue Samples**

The current study was authorized by the Research Ethics Committee of Navy General Hospital of Chinese PLA, China. All patients agreed to the procedure and signed consent forms. All specimens were handled and made anonymous per the ethical and legal standards.

In this retrospective study, a total of 100 paired formalin-fixed and paraffin-embedded cancerous and adjacent noncancerous tissues were obtained from thyroid cancer patients who underwent total thyroidectomy at the Department of General Surgery, Navy General Hospital of Chinese PLA, between January 2008 and December 2013. The diagnosis of thyroid cancer was confirmed by pathologic examination after surgical operation. There were 30 men and 70 women, ranging in age from 28 to 76 years (median age, 50 years). Tumor stage was performed according to the sixth edition of the TNM (tumor, node, metastasis) classification of the International Union Against Cancer. Patient characteristics—including age, sex, tumor size, histologic type, TNM stage, presence of extrathyroidal invasion, lymph nodes, and distant metastases—are summarized in Table 1.

**Cell Culture**

Human papillary thyroid cancer cell lines BCPAP and K1 were purchased from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). All cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, California) supplemented with 10% fetal calf serum (GIBCO, Waltham, Massachusetts) in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C.

**Immunohistochemistry**

 Archived formalin-fixed, paraffin-embedded tissue blocks were retrieved, reviewed by the pathologist, and cut to provide 4-μm-thick sections for immunohistochemistry. Tissue sections were all deparaffinized with xylene, rehydrated, and subjected to microwave antigen retrieval in citrate buffer (pH 6.0) for 30 minutes. The endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide in methanol. Then, the sections were incubated with the monoclonal anti-FGF19 antibody (1:500 dilution, SC-73984; Santa Cruz Biotechnology, Santa Cruz, California) at 4°C overnight and incubated for 30 minutes with the biotinylated secondary antibody after being washed with Tris-buffered NaCl solution for 30 minutes. After that, the sections were washed in phosphate-buffered saline, and immunoreactivity was visualized with a Dako Envision Kit (Dako Cytomation, Carpinteria, California) based on the manufacturer’s instructions. For negative control, no immunoreactivity was observed in tissue sections where the primary antibody was replaced by isotype-matched irrelevant antibody. For positive control, the thyroid cancer tissues with the overexpression of FGF19 protein were confirmed by Western blot.

To evaluate the immunostaining of FGF19 protein, all sections were scored by 2 independent pathologists who were blinded to patients’ characteristics, and any discrepancies were resolved by consensus. Sections were scored as positive if epithelial cells showed immunopositivity in cytoplasm with or without plasma membrane. These sections were scored with a semiquantitative scoring system combining the staining intensity with the percentage of positive cells as described by the previous studies. The intensity was classified as follows: 0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of positive cells was judged according to Remmele and Stegner’s criteria: 1, 0% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, >75%. A final immunoreactive score was achieved by multiplying the intensity and percentage of positive cells. To divide all 100 patients with thyroid cancer into high and low FGF19 expression groups, the median value of FGF19 protein immunoreactive score was used as a cutoff point.

**RNA Interference of FGF19**

BCPAP and K1 cells during the logarithmic growth phase were transfected with nontargeting negative-control small interfering RNA (siRNA; AMBIION, Austin, Texas) and human FGF19-specific siRNA (AMBIION) with liposome
(Lipofectamine 2000; Invitrogen) according to the manufacturer’s instructions. Cells were verified and used for analysis 48 hours after transfection.

**Western Blot Analysis**

Western blot analysis was performed to detect the expression levels of FGF19 protein in BCPAP and K1 cells harvested 48 hours after transient transfection with the monoclonal anti-FGF19 antibody (1:100 dilution, SC-73984; Santa Cruz Biotechnology) and secondary antibody (Santa Cruz Biotechnology) as described previously.\(^{18,19}\) Equal protein sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody (Santa Cruz Biotechnology), which was used as an internal control for the normalization of candidate proteins. Protein expression was visualized by enhanced chemiluminescence and exposure to chemiluminescent film (Amersham, Buckinghamshire, UK). Band intensities were quantified by densitometry.

**Cell Migration and Invasion Assays**

Cell migration assay was performed to detect the migration abilities of BCPAP and K1 cells transfected with FGF19 siRNA and nontargeting negative-control siRNA with Transwell chambers (8.0-μm pore size; Millipore, Billerica, Massachusetts) in 24-well plates. In brief, transfected cells were starved overnight and then seeded in the upper chamber at a density of 2 \( \times 10^5 \) cells/mL in 400 μL of medium containing 0.5% FBS (fetal bovine serum). Medium with 5% FBS was added into the lower chambers as a chemoattractant. Following a 24-hour incubation at 37°C with 5% CO\(_2\), nonmigrating cells in the upper chamber were removed with a cotton swab, and migrating cells were fixed in 100% methanol and stained with 0.5% crystal violet in 2% ethanol. The number of migrating cells were manually counted at 200× magnification from 10 different fields of each filter.

Cell invasion assay was performed to detect the invasive abilities of BCPAP and K1 cells transfected with FGF19 siRNA and nontargeting negative-control siRNA via Transwell chambers (8.0-μm pore size; Millipore) coated with Matrigel (4× dilution, 60 μL/well; BD Bioscience, Franklin Lakes, New Jersey) in 24-well plates. In brief, transfected cells were resuspended in 200-μL serum-free 1640 medium (Invitrogen) and placed into the upper chamber of the insert with Matrigel.

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**Table 1. Association of FGF19 Expression with Various Clinicopathologic Features of Patients with Thyroid Cancer.**

<table>
<thead>
<tr>
<th>Clinicopathologic Features</th>
<th>Cases, n</th>
<th>FGF19 Expression, n (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45</td>
<td>42</td>
<td>22 (52.4)</td>
<td>20 (47.6)</td>
</tr>
<tr>
<td>≥45</td>
<td>58</td>
<td>30 (51.7)</td>
<td>28 (48.3)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>15 (50.0)</td>
<td>15 (50.0)</td>
</tr>
<tr>
<td>Female</td>
<td>70</td>
<td>37 (52.9)</td>
<td>33 (47.1)</td>
</tr>
<tr>
<td>Tumor size, cm</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;2</td>
<td>72</td>
<td>35 (48.6)</td>
<td>37 (51.4)</td>
</tr>
<tr>
<td>≥2</td>
<td>28</td>
<td>17 (60.7)</td>
<td>11 (39.3)</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular carcinoma</td>
<td>48</td>
<td>25 (52.1)</td>
<td>23 (47.9)</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic variant</td>
<td>42</td>
<td>22 (52.4)</td>
<td>20 (47.6)</td>
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<tr>
<td>Tall cell variant</td>
<td>10</td>
<td>5 (50.0)</td>
<td>5 (50.0)</td>
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<td>TNM stage</td>
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<td>.008</td>
</tr>
<tr>
<td>I–II</td>
<td>32</td>
<td>7 (21.9)</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>III–IV</td>
<td>68</td>
<td>45 (66.2)</td>
<td>23 (33.8)</td>
</tr>
<tr>
<td>Extrathyroidal invasion</td>
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<td>.01</td>
</tr>
<tr>
<td>Negative</td>
<td>70</td>
<td>30 (42.9)</td>
<td>40 (57.1)</td>
</tr>
<tr>
<td>Positive</td>
<td>30</td>
<td>22 (73.3)</td>
<td>8 (26.7)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td>.01</td>
</tr>
<tr>
<td>Absent</td>
<td>48</td>
<td>16 (33.0)</td>
<td>32 (67.0)</td>
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<tr>
<td>Present</td>
<td>52</td>
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<tr>
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<td>62</td>
<td>22 (35.5)</td>
<td>40 (64.5)</td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>30 (78.9)</td>
<td>8 (21.1)</td>
</tr>
</tbody>
</table>

Abbreviations: FGF19, fibroblast growth factor 19; NS, nonsignificant; TNM, tumor, node, metastasis.
Medium with 5% FBS was added into the lower chambers as a chemoattractant. Following a 24-hour incubation at 37°C with 5% CO₂, cells remaining on the upper membrane were carefully removed. The number of invasive cells were manually counted at 200× magnification from 10 different fields of each filter.

All assays were performed in triplicate, and the mean values were calculated.

**Statistical Analysis**

Data were expressed as mean ± SD and subjected to statistical analysis via SPSS 13.0 software (IBM Inc, Chicago, Illinois). Comparison among different groups was analyzed with paired samples t tests. Differences in ≥2 proportions were calculated with Fisher’s exact test and chi-square. Data were considered significant at P < .05.

**Results**

**Increased Expression of FGF19 Protein in Human Thyroid Cancer Tissues**

Positive immunostaining of FGF19 protein expression was localized in cytoplasm with or without membrane of malignant cells, as shown in Figure 1A. It was observed in 82 (82.0%) of 100 patients with thyroid cancer, while it was seen faintly or with no staining in adjacent noncancerous tissues, as shown in Figure 1B. Statistically, the expression level of FGF19 protein in thyroid cancer tissues was significantly higher than that in normal tissues (P < .001; Figure 1C).

Based on our immunohistochemistry scoring system, an optimal cutoff value (3.88) was identified: for the high FGF19 expression group, the FGF19 expression level was higher than the cutoff value (median expression value, 4.02; n = 52, 52.0%); for the low FGF19 expression group, the FGF19 expression level was lower than the cutoff value (median expression value, 2.96; n = 48, 48.0%).

**Increased Expression of FGF19 Protein Associates with Aggressive Progression of Thyroid Cancer Patients**

The association of FGF19 protein expression with clinicopathologic parameters of patients with thyroid cancer is summarized in Table 1. FGF19 overexpression was significantly associated with advanced TNM staging (P = .008), presence of extrathyroidal invasion (P = .01), lymph nodes metastasis (P = .01), and distant metastasis (P = .02). However, no significant associations were found between FGF19 expression and age, sex, tumor size, and histologic type (all P > .05; Table 1).

**Knocking Down FGF19 Expression Suppresses the Migration and Invasion Potentials of Thyroid Cancer Cell Lines**

The above statistical analysis showed that FGF19 overexpression was significantly associated with lymph node metastasis, which prompted us to further confirm whether FGF19 plays any direct role in enhancing the migration and invasion abilities of thyroid cancer cells. As shown in Figure 2, the expression levels of FGF19 protein in 2 transfected thyroid cancer cell lines were both significantly lower than those in the control cells (both P < .001).
Interestingly, the results indicated that the migratory activities of FGF19 siRNA–transfected BCPAP and K1 cells were both dramatically reduced and that loss of FGF19 expression in both cell lines with FGF19 siRNA also inhibited the cell invasion potentials in the Matrigel substrate (all $P < .01$, Figure 3).

**Discussion**

A better understanding of the molecular mechanisms underlying thyroid carcinogenesis, in addition to rapid advancements in molecular diagnostic technology, has made a distinct progress in the research field of diagnostic, prognostic, and therapeutic options for patients with thyroid cancer. The identification of biomarkers useful for thyroid cancer diagnosis and for the early detection of metastatic cases is of great clinical significance to improve the clinical outcome of patients with this disease. In the current study, our data provided evidence that FGF19 was specifically expressed in primary thyroid cancer tissues and that its immunoreactivity was frequently stronger in cancerous tissues than in their adjacent noncancerous tissues, suggesting that FGF19 might help distinguish benign thyroid from malignant tumors. Then, our statistical analysis demonstrated that FGF19 expression levels were often increased in thyroid cancer tissues with aggressive clinicopathologic features, implying its potential as a marker for tumor aggressiveness. More important, our functional studies demonstrated that FGF19 knockdown was associated with a suppression in cellular migration and invasion in 2 human thyroid cancer cell lines tested. To the best of our knowledge, this is the first study showing the clinical significance of FGF19 in human thyroid cancer and its oncogenic roles in promoting the motility of human cancer cells.

FGFs act as humoral local factors and play diverse biological roles in regulating cell growth and differentiation. Among FGFs, FGF19, with FGF21 and FGF23, belongs to the endocrine FGF subfamily, which circulates in serum and acts in an endocrine-like manner. Different from the classical FGFs, FGF19 has a much-reduced heparan sulfate proteoglycan–binding affinity that allows it to function as endocrine hormone. FGF19 selectively binds to FGFR4 and is a major metabolism regulator under normal physiological conditions. Interestingly, FGF19 signaling pathway also contributes to the development and progression of cancer. FGF19 overexpression has been observed in a variety of human cancer types, including lung squamous cell carcinoma, hepatocellular carcinoma, colon adenocarcinoma, and prostate cancer. Functionally, it exerts proliferative and chemotactic effects on malignant cells and extends cell survival; it stimulates tumor angiogenesis; and it increases

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**Figure 3.** Knocking down FGF19 expression inhibits the migration and invasion of BCPAP (A) and K1 (B) cells in vitro. *$P < .05$ and **$P < .01$, respectively, when compared with control cells. FGF19, fibroblast growth factor 19; si-FGF19, cells transfected with FGF19 small interfering RNA; si-con, nontargeting negative control small interfering RNA.
motility and adhesion. In line with these existing evidences, our study here also demonstrated the overexpression of FGF19 in human thyroid cancer tissues and found the significant associations between high FGF19 expression and tumor aggressive progression. To assess the importance of FGF19 in tumor cell motility, a FGF19-specific siRNA was utilized to selectively inhibit the expression of FGF19, which demonstrated its suppressive role in the migration and invasion abilities of human thyroid cancer cells.

In conclusion, our data revealed that the increased expression of FGF19 might be involved in the malignant behaviors of thyroid cancer, highlighting its potentials as a molecular marker for early diagnosis and as a potential target for therapeutic intervention of this disease. While the aforementioned data imply the roles of FGF19 in thyroid carcinogenesis, the molecular mechanism by which it functions in this manner remains unknown. Further studies are required to clarify this problem.

Author Contributions

Xiliang Zhang, substantial contributions to the conception, design of the work; the acquisition, analysis, interpretation of data for the work; and drafting the work and revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved; Zhonghua Wang, substantial contributions to the conception, design of the work; the acquisition, analysis, interpretation of data for the work; and drafting the work and revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved; Lei Tian, substantial contributions to the conception, design of the work; the acquisition, analysis, interpretation of data for the work; and drafting the work and revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved; Jiapeng Xie, substantial contributions to the conception, design of the work; the acquisition, analysis, interpretation of data for the work; and drafting the work and revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved; Futing Jiang, substantial contributions to the conception, design of the work, the acquisition, analysis, and interpretation of data for the work; and drafting the work, revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Disclosures

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Sponsorships: None.
Funding source: None.

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