Association of Cancer Stem Cell Markers With Aggressive Tumor Features in Papillary Thyroid Carcinoma
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Background: Identifying accurate prognostic molecular markers for papillary thyroid carcinoma (PTC) is important because many patients with PTC may be erroneously considered to have low-risk tumors. Evidence is also accumulating to support the existence of cancer stem cells in PTC.

Methods: Thirty controls and 167 patients with PTC were selected to establish a tissue microarray to investigate cancer stem cell marker expression in samples from an established pathological database. The protein expressions of CD44, CD133, epithelial cell adhesion molecule (EpCAM), CD45, and CD90 were evaluated by immunohistochemical assay in the tissue microarray.

Results: The protein levels of CD44, CD133, and EpCAM were significantly increased in PTC tissue compared with tissue from the controls. A positive correlation was found between cancer stem cell markers and tumor, node, and metastasis staging.

Conclusions: Among a subset of patients with PTC, cancer stem cells detected by immunohistochemistry can be used as prognostic markers to screen for potential tumor dissemination. Whether these cancer stem cell markers are potentially therapeutic targets — and, thus, could be used for effective adjuvant treatment strategies — remains to be seen, and more data are needed.

Introduction
Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy, and an estimated 62,450 new cases of thyroid cancer will be diagnosed in 2015 in the United States alone; of these diagnoses, 3 out of 4 cases will occur in women.1,2 In general, PTC has a favorable prognosis, the overall 10-year survival rate of patients with PTC is about 90%, however, approximately 10% and 20% of patients with stage 1 or 2 PTC, respectively, have disease recurrence.2 Tumor recurrence affects the quality of life of patients; among those who develop local recurrence, some will die from the disease, indicating the existence of more aggressive variants of PTC. Up to 30% of patients with differentiated thyroid carcinoma may have tumor recurrence within several decades; 66% of recurrences occur within the first decade following initial therapy.3 Therefore, identifying the subpopulation at high-risk for disease recurrence is necessary.

Based on clinical and pathological studies, various factors, such as age, sex, tumor size, extrathyroidal extension, and distant metastasis, have been investigated to determine whether an association exists between them and PTC recurrence; however, the matter is controversial.4 Molecular and cellular events have been shown to be associated with PTC, and the cancer progenitor/stem cell is considered by some as a common cellular alteration detected in patients with PTC.5,6 Cancer stem cells, a small subpopulation of cells with stem-like properties, are key factors for tumor initiation and recurrence.7 Evidence supporting the existence of cancer stem cells in PTC is rapidly accumulating.8-12

Recent research indicates that increased cancer stem cell–like features play an important role in the progression of PTC.13 The cancer stem cell theory was established based on the observation that cancer cell populations are heterogeneous.14 The identification and characterization of cancer stem cells in tumors largely depend on the surface markers shared with normal stem cells.15 These populations have been identified and isolated using the surface markers CD44 and CD24 in a human PTC cell line, TPC-1 (BHP10-3).16 Research shows that POU5F1, a stem cell marker, is significantly higher in CD44+ (high levels of CD44) and CD24 Lin (low levels of CD24) cells, supporting the hypothesis that CD44+CD24 Lin− cells are potential cancer
stem cells. Further study suggests that CD44+CD24−Lin− PTC cells can form thyrospheroids, which are important in vitro features of cancer stem cells; moreover, data indicate that thyrospheroids can initiate a tumor in immunodeficient mice. Several other potential stem cell markers have been used by others in thyroid cancer stem cell research, such as CD133 and epithelial cell adhesion molecule (EpCAM).

Although the prognostic influence of cancer stem cells in PTC is unclear, the use of cancer stem cell markers may help histopathologically determine the existence of cancer stem cells in PTC tissue, possibly impacting patient prognosis. Traditional immunohistochemical examination consists of single-sectioning paraffin blocks and is amenable to routine pathology; however, a wide variety of the staining condition by traditional assay may limit its utility for detecting cancer stem cell markers in resected PTC samples. Tissue microarray is a high-throughput technology used for analyzing molecular markers in oncology. It offers added benefit with respect to decreased technical variability during the staining and interpretation process.

In our study, we established a tissue microarray process to investigate the expressions of cancer stem cell markers in tissue samples from a pathological database of patients with PTC. The specimens used in the tissue microarray consisted of different pathological stages of PTC and were stained by immunohistochemical assay. The protein expressions of CD44, CD133, EpCAM, CD45, and CD90 were then evaluated for each pathological stage of PTC. Furthermore, we set forth to determine the correlation between cancer stem cell markers and the pathological stages of PTC.

Materials and Methods
Thyroid nodule tissue from 670 patients was collected from a pathological database maintained by the Third Affiliated Hospital of Wenzhou Medical University (Ruian, Zhejiang, China). All specimens with a diagnosis of PTC were collected following surgical excision.

In this retrospective study, we reviewed the database for patients undergoing thyroid nodule resection between August 2012 and September 2013. This study was approved by the Institutional Review Board at Wenzhou Medical University.

Patient Selection
Patients were excluded if they had radiotherapy, chemotherapy, or both, either preoperatively or postoperatively. Patients also met exclusion criteria if they had certain types of conditions, including autoimmune disease, cerebrovascular accident, coronary artery disease, viral hepatitis, or another type of cancer. Overall, 167 patients postoperatively diagnosed with PTC, along with 30 benign, non-PTC controls, met inclusion criteria based on the completeness of their data. Dates of diagnoses and collection times ranged from August 2012 to September 2013.

Patients were divided into 4 treatment groups according to tumor, node, and metastasis (TNM) staging per the classification of thyroid cancer by the American Joint Committee on Cancer. PTC stage 1 was defined as papillary carcinoma localized to the thyroid gland. PTC stage 2 was defined as either (a) papillary carcinoma that has spread distantly in patients younger than 45 years, or (b) papillary carcinoma larger than 2 cm but no larger than 4 cm, and was limited to the thyroid gland in patients older than 45 years. PTC stage 3 was defined as papillary carcinoma occurring in patients older than 45 years that was larger than 4 cm and was limited to the thyroid or with minimal extrathyroidal extension, or positive lymph nodes limited to the pretracheal, paratracheal, or prelaryngeal/Delphian nodes. PTC stage 4 was defined as papillary carcinoma in patients older than 45 years that extended beyond the thyroid capsule to the soft tissues of the neck, cervical lymph node metastases, or distant metastases.

Of the 167 patients, 139 (83.2%) were women and 28 (16.8%) were men. Of the non-PTC controls with resected thyroid nodules, 26 (86.6%) were women and 4 (13.3%) were men. The mean age of the 167 study patients was 50.21 ± 11.65 years compared with 48.9 ± 11.34 years for the controls. Of the patients with PTC, 11 had a family history of diabetes mellitus, 26 had a family history of hypertension, 24 had a history of Hashimoto thyroiditis (chronic lymphocytic thyroiditis), and 3 were former smokers; by contrast, no controls had family histories of diabetes mellitus, hypertension, and smoking, and 1 had a history of Hashimoto thyroiditis history. Medical records were also reviewed (Table 1).

Tissue Microarrays
The surgical resection of PTC samples provided ample tissue for paraffin-embedded tissue blocks, and tissue array was constructed from the paraffin blocks. Prior to constructing the tissue arrays, the blocks were categorized into 5 groups: non-PTC ("benign controls") and stages 1 to 4. The categorization was made according to each study patient's clinical history and diagnosis.

To avoid sample-selection bias, 2 pathologists reviewed the hematoxylin and eosin–stained tissue sections to identify areas of PTC and select the regions in blocks as donor core tissues. The reviewers were blinded to the study patients' clinical history. Donor core tissues with a diameter of 1 mm were obtained from corresponding regions in the donor paraffin block and embedded into a recipient block. Cores from archived specimens of normal thyroid tissue adjacent to
Computer Image Analysis

Computer image analysis was performed to quantify the expressions of the cancer stem cell markers in 167 study patients with PTC and 30 benign controls. Two pathologists performed a pathological reading to confirm the PTC diagnosis for each tissue microarray slide before the digital image was captured. The imaging fields were randomly chosen from each core tissue to ensure sampling objectivity. Five imaging fields were scanned for each specimen sample. All digital images were acquired with the Olympus Microscope IX51 (Olympus, Pittsburgh, Pennsylvania) at 200 magnification using the Spot camera via the Image-Pro Plus Imaging System (Media Cybernetics, Warrendale, Pennsylvania) and stored as jpg data files (resolution was fixed at 200 pixels per inch).

The acquired color images from immunohistochemical staining were defined as standard threshold according to software specifications. The software quantified the threshold area represented in these images. Either the PTC images or normal thyroid images were delineated from the obtained tissues as a region of interest (ROI) using the set color threshold subroutine of the image analysis software. Because of the existence of the duct structure of PTC — in particular, the benign thyroid tissue — the “no tissue” regions were excluded within the ROI to avoid faulty measurement.

The integrated optical density measured for positive staining was a representation of the mass and a measurement of the total amount of positive staining in the delineated regions. Values of integrated optical density were normalized by dividing by the area of the threshold ROI of the positive staining. Positive expression was represented as the digital values of the integrated optical density/area, allowing us to analyze the statistical significance among the study groups.

Statistic Analysis

Analysis of variance was performed using SPSS16.0 (IBM, New York, New York) to determine the statistical significance of the level of expression of the cancer stem cell markers, if any, between TNM staging and controls. Spearman rank correlation coefficient was performed to analyze the correlation between cancer stem cell markers and TNM staging. Values are reported as mean plus or minus standard deviation. Differences between groups were regarded as statistically significant when \( P \) values were less than .05.

Results

Protein Expressions of CD44, CD133, EpCAM, CD45, and CD90

The expression pattern of CD44 showed that the positive staining mainly presented on the cell
membrane of PTC cells. Strong staining of CD44 was found in all PTC tissue, but weak and negative staining were both found in the tissue from the benign controls. Most PTC cells in stages 3 and 4 with strong CD44 staining also showed cytomorphological features of poor polarity, which was characterized by the loss of both apical-basal polarity and well-arranged order of neighboring cells. By contrast, PTC cells in stages 1 and 2 had cytomorphologic features of well polarity with weaker staining of CD44. The computer quantification of CD44 expression in control specimens was 56.4 ± 29.88. Compared with the control specimens, significant increases of CD44 expression were seen in specimens with stage 1 (241.71 ± 87.25; P < .01), stage 2 (311.98 ± 135.26; P < .01), stage 3 (354.71 ± 103.42; P < .01), and stage 4 (374.58 ± 51.6; P < .01; Fig 1).

The expression pattern of CD133 showed that the positive staining mainly presented in the cytosol of PTC cells. Similar to CD44 expression, strong staining of CD133 was found in all PTC tissue, but weak and negative staining was found in the tissue from the benign controls. The PTC cells from stages 3 and 4 tissue samples had poor polarity with strong CD133 staining, whereas the PTC cells from stages 1 and 2 tissue samples had well polarity with weaker staining of CD133. The computer quantification of CD133 expression in the control specimens was 52.64 ± 37.45. Compared with the controls, CD133 expression slightly increased in the PTC tissue samples with stage 1 (152.2 ± 52.72; P > .05) but significantly increased in the PTC tissue samples with stage 2 (201.18 ± 60.33; P < .01), stage 3 (242.49 ± 66.81; P < .01), and stage 4 (284.81 ± 87.63; P < .01; Fig 2).

EpCAM showed a similar expression pattern as CD133, in which the positive staining mainly presented in the cytosol of PTC cells. Strong staining of EpCAM was seen in all PTC malignant tissue samples but weak and negative staining was seen in the benign controls. The computer quantification of EpCAM expression in control specimens was 48.30 ± 27.07. Compared with the controls, EpCAM expression increased in the stage 1 (157.48 ± 56.48; P > .05) PTC tissue samples but significantly increased in the PTC tissue samples with stage 2 (191.33 ± 51.12; P < .01), stage 3 (201.83 ± 58.20; P < .01), and stage 4 (226.75 ± 81.44; P < .01; Fig 3). The expressions of CD45 and CD90 showed weak (CD45) and mild staining (CD90) in the PTC tissue samples from all 4 stages as well as the tissue samples from the benign controls. Analysis of variance did not indicate a statistical significance among the TNM stages and controls (Table 2).

**Correlation of CD44, CD133, and EpCAM**

We further analyzed the correlation between the 3 cancer stem cell markers (CD44, CD133, and EpCAM) and TNM staging. Positive correlations were

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**Table 2.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>CD44 Expression (IOD/area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
</tr>
<tr>
<td>Benign</td>
<td>800</td>
</tr>
</tbody>
</table>

**Fig 1A–F.** — Detection of CD44 expressions by immunohistochemistry and computer image analysis of positive staining in the papillary thyroid carcinoma tissue of TNM stages as well as in the tissue of benign controls. (A) Stage 1 tumor. (B) Stage 2 tumor. (C) Stage 3 tumor. (D) Stage 4 tumor. (E) Benign controls. (F) CD44 expression.

*P < .05 vs benign controls.
IOD = integrated optical density, TNM = tumor, lymph node, metastasis.
Fig 2A–F. — Detection of CD133 expression by immunohistochemistry and computer image analysis of positive staining in the papillary thyroid carcinoma tissue of 4 TNM stages and benign controls. (A) Stage 1 tumor. (B) Stage 2 tumor. (C) Stage 3 tumor. (D) Stage 4 tumor. (E) Benign controls. (F) CD44 expression.

*P < .05 vs benign controls. **P < .05 vs stage 1.

IOD = integrated optical density, TNM = tumor, lymph node, metastasis.

EpCAM = epithelial cell adhesion molecule, IOD = integrated optical density, TNM = tumor, lymph node, metastasis.
found between CD44 and TNM staging ($r^2 = 0.5138; P < .01; \text{Fig } 4\text{A}$), between CD133 and TNM staging ($r^2 = 0.6107; P < .01; \text{Fig } 4\text{B}$), and between EpCAM and TNM staging ($r^2 = 0.27; P < .01; \text{Fig } 4\text{C}$).

### Discussion
The results from this study demonstrate significant increases of expressions of CD44, CD133, and EpCAM in PTC tissue samples compared with the tissue samples from the benign controls. The increased amounts of these cancer stem cell surface markers were moderate in stage 1 but were the most pronounced in stages 2 to 4 of PTC. To our knowledge, this is the first study to examine the relationship between cancer stem cell surface markers and TNM staging of PTC.

To date, CD44, CD133, and EpCAM, which were initially described as surface markers for malignant progenitor cells, have been widely explored as reliable markers for isolating cancer stem cells in many tumor types.\textsuperscript{20} Interest in these cancer stem cell markers has increased with the observation that the overexpression of cancer stem cell surface markers in tumor cells is associated with therapeutic failure and tumor recurrence.\textsuperscript{21} In PTC, immunohistochemical staining of CD44 has been shown to have a strong membranous expression pattern, which is consistent with our results.\textsuperscript{22} In addition, other studies show that the protein levels of CD44 significantly increase in biopsy tissue samples of most patients with PTC.\textsuperscript{22-24} Accordingly, in our study, the overall expression of CD44 was increased among all patients with PTC.

Research has shown that a CD133-positive subpopulation, such as cancer-initiating cells, can reproduce an original tumor and serially transplant it for several generations in immunodeficient mice, whereas CD133-negative cells cannot form tumors.\textsuperscript{25} Despite CD133 being known as a cancer stem cell marker in many tumors and tissue, CD133 expression in PTC was detected at a lower level in PTC cell lines and in patients with PTC; however, it was detected at a higher level in patients with anaplastic thyroid carcinoma, which is a highly aggressive neoplasm.\textsuperscript{26-28} With regard to subcellular compartmentalization, we found CD133 expression to be predominantly cytoplasmic, which is consistent with previous observations.\textsuperscript{28} In our observations, very weak CD133 expression was detected in normal human thyroid tissue, and a lower level of CD133 expression was detected in patients with stages 1 and 2 PTC. However, a high level of CD133 expression was detected in patients with stages 3 and 4 PTC. Cytomorphological features of stages 3 and 4 PTC showed poor polarity, which is consistent with its aggressive growing pattern, in which tumor growth was found beyond the thyroid capsule, including metastasis to the soft tissues of the neck, cervical lymph node metastases, and distant metastases.

Although lymphatic spread is the most common form of PTC progression, the existing residual micrometastatic tumor cells in fact contribute to the

### Table 2. — Expressions of CD45 and CD90 in Study Patients With Papillary Thyroid Carcinoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of Patients</th>
<th>Intensity of IOD (mean ± SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD45</td>
<td>CD90</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 45</td>
<td>57</td>
<td>54.43 ± 60.28</td>
<td>1.63 ± 2.07</td>
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<tr>
<td>≥ 45</td>
<td>110</td>
<td>65.14 ± 79.93</td>
<td>1.74 ± 2.34</td>
</tr>
<tr>
<td>Sex</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>28</td>
<td>60.47 ± 66.1</td>
<td>2.38 ± 2.75</td>
</tr>
<tr>
<td>Female</td>
<td>139</td>
<td>61.69 ± 75.48</td>
<td>1.57 ± 2.11</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>101</td>
<td>59.95 ± 71.31</td>
<td>1.76 ± 2.26</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>81.30 ± 103.95</td>
<td>1.00 ± 1.24</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>60.23 ± 72.06</td>
<td>1.66 ± 2.22</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>45.75 ± 37.97</td>
<td>3.36 ± 4.36</td>
</tr>
<tr>
<td>Controls</td>
<td>30</td>
<td>8.14 ± 7.7</td>
<td>1.41 ± 1.73</td>
</tr>
</tbody>
</table>

IOD = integrated optical density, SD = standard deviation, TNM = tumor, lymph mode, metastasis.

![Figure 4A–C](image-url) — Positive correlation of (A) CD44, (B) CD133, and (C) EpCAM expression in TNM staging. EpCAM = epithelial cell adhesion molecule, TNM = tumor, lymph mode, metastasis.
Residual, micrometastatic tumor cells are undetectable by conventional histopathological examination. Previously, immunohistochemistry using the monoclonal anti-EpCAM antibody (Ber EP4) for visualizing disseminated tumor cells in “tumor free” lymph nodes was performed by others. In that study, positive EpCAM tumor cells were revealed among “tumor-free” lymph nodes in 12.5% of cases. EpCAM is a 40kDa transmembrane glycoprotein frequently overexpressed in cancer progenitor cells, which are cancer-initiating cells in many human malignancies, including PTC and — although at very lower levels — in normal epithelia. Thus, the finding of positive EpCAM expression in tumor cells among “tumor free” lymph nodes may be clinically significant in relation to increased risk of local recurrence and distant metastases. However, a follow-up analysis (mean observation, 72 months) indicated no prognostic significance in cases with positive EpCAM expression because these patients underwent treatment of radioiodine, which can eliminate microdisseminated tumor residue. Nevertheless, detection of EpCAM is clinical significant. The concept is supported by 2 studies that identified the nucleus localization of EpCAM as an aggressive marker for poor prognosis among patients with thyroid cancer.

In our study, the pattern of EpCAM expression showed weak staining in normal human thyroid tissue and strong staining in PTC tissue, with the staining increasing from stage 1 to stage 4. We also found that increased levels of cancer stem cell markers (CD44, CD133, and EpCAM) were positively related to the TNM staging of PTC. However, future studies are needed to define the specificity of cancer stem cell markers in PTC.

Conclusions

Identification of accurate prognostic molecular markers for papillary thyroid carcinoma (PTC) is important because most patients with PTC may be erroneously diagnosed as having low-risk tumors. Our findings suggest that, compared with traditional histopathological examination, tissue-array based immunohistochemistry for cancer stem cell marker detection can more accurately evaluate the biological characteristics of PTC. Thus, locating cancer stem cells in a subset of patients with PTC may be molecular markers that can be used as prognostic markers and to screen for tumor dissemination. However, whether these cancer stem cells markers are potential therapeutic targets for effective adjuvant treatment strategies requires additional evaluation.

References