Original Article

Correlation between calcification and bone sialoprotein and osteopontin in papillary thyroid carcinoma

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Abstract: The correlation between calcification and papillary thyroid carcinoma has received increasing attention. We investigated the ability of bone sialoprotein (BSP) and osteopontin (OPN) protein levels to diagnose papillary thyroid carcinoma (PTC), and explored the correlation between BSP and OPN protein levels and calcification in PTC. Archival PTC specimens from patients with PTC with calcification and lateral cervical lymph node metastasis (LNM) were included in this retrospective immunohistochemical study. The protein levels of BSP and OPN were analysed immunohistochemically using routinely prepared tissue sections. PTC specimens from 66 patients with PTC were reviewed retrospectively (25 patients with histological calcification seen in paraffin sections, 41 patients without calcification; 35 patients with lateral cervical LNM, 31 patients without LNM). The percentage of samples that had cells that demonstrated positive protein staining differed significantly between PTC specimens, benign thyroid nodules, and adjacent normal follicular epithelium (BSP: 87.88%, 55.00%, and 42.50%, respectively; OPN: 83.33%, 70.00% and 50.00%, respectively). There was a significant difference in the immunohistochemical score (IHS) for BSP and OPN protein staining between PTC specimens with and without calcification (P < 0.05). The level of BSP protein staining was found to be significantly correlated with the level of OPN protein staining in PTC specimens. We conclude that the strong correlation between BSP and OPN and PTC suggests a role for BSP and OPN in calcification and tumor progression of PTC. BSP and OPN might be useful tumour markers for the diagnosis of PTC with limited value, because both of them had low specificity.

Keywords: Papillary thyroid carcinoma, immunohistochemistry, bone sialoprotein (BSP), osteopontin (OPN), calcification

Introduction

Differentiated thyroid carcinoma (DTC), with a rapidly increasing incidence, is the most common endocrine malignancy, but with generally favorable survival [1]. Between 1996 and 2008, an increasing incidence of papillary thyroid carcinoma (PTC) was reported in Denmark, with age standardized incidence rates increasing from 1.43 per 100,000 per year in 1996 to 2.16 per 100,000 per year in 2008 [2]. PTC is the most common type of DTC [3, 4]. With the widespread use of high-frequency ultrasound and in-depth research on thyroid calcification, correlations between calcification and PTC have received increasing attention [5, 6]. Thyroid calcification can occur in both malignant and benign thyroid disease, but a higher percentage of nodules complicated by calcification has been described in thyroid cancer compared with benign nodules [7].

Observations from other nonthyroid experimental models and clinical studies have illustrated the critical role of bone sialoprotein (BSP) in tumour progression and metastasis. The recent discovery that BSP can be detected in a variety of human cancers, particularly those that metastasize preferentially to the skeleton, shed light on potential new biological functions for this protein [8]. Osteopontin (OPN) expression also plays an important role in tumorigenesis, tumor progression and metastasis [9]. Previous study reported the broad distribution of OPN in
Calcification and BSP/OPN in PTC

Table 1. IHS of BSP protein staining of PTC (n = 66)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Immunohistochemical score</th>
<th>Positive n (%)</th>
<th>Statistical significance</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PTC tissue</td>
<td>66</td>
<td>8</td>
<td>13</td>
<td>29</td>
</tr>
<tr>
<td>Benign nodules</td>
<td>40</td>
<td>18</td>
<td>4</td>
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<tr>
<td>Normal tissue</td>
<td>40</td>
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Histological calcification seen in paraffin sections

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<thead>
<tr>
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<th>Negative</th>
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<tbody>
<tr>
<td>with calcification</td>
<td>25 (100.00)</td>
<td>41 (80.49)</td>
</tr>
<tr>
<td>without calcification</td>
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Calcification on B-ultrasound

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
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<tr>
<td>with calcification</td>
<td>43 (90.70)</td>
<td>23 (82.61)</td>
</tr>
<tr>
<td>without calcification</td>
<td></td>
<td></td>
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</tbody>
</table>

LNM

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>35 (88.57)</td>
<td>31 (87.10)</td>
</tr>
<tr>
<td>Negative</td>
<td>31 (87.10)</td>
<td>27 (87.10)</td>
</tr>
</tbody>
</table>

Data presented as the number of specimens with the following 'immunohistochemical score': - negative; + weakly positive; ++ moderately positive; +++ strongly positive. *PTC tissue versus benign nodules, Z = -5.721; two-tailed two-independent samples test. †PTC tissue versus normal tissue, Z = -6.356; two-tailed two-independent samples test. ‡with calcification versus without calcification in paraffin sections, Z = -2.534; two-tailed two-independent samples test. §with calcification versus without calcification on B-ultrasound, Z = -0.591; two-tailed two-independent samples test. ¶LNM negative versus benign nodules, Z = -1.318; two-tailed two-independent samples test.

The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of Huashan Hospital, Fudan University (no. 2013 M-002). As only routine procedures were used on archival surgical specimens and no treatment interventions were investigated, patients providing samples for the study were not required to provide written or verbal informed consent.

Immunohistochemical analysis

All surgical specimens were subjected to routine pathological examination and immunohistochemical staining for BSP and OPN proteins. The tissue samples were fixed in 30% formalin, dehydrated in ethanol, and embedded in paraffin wax. For routine pathological examination, all specimens were sliced continuously into 4-µm sections, stained with haematoxylin and eosin, and examined by two independent pathologists (Zhongwen Zhou and Feng Tang) with experience in thyroid pathology. These examinations were conducted blindly and independently, and the pathologists did not know the original histological diagnosis. All speci-
mens were classified according to the published diagnostic criteria for thyroid tumours [11].

Immunohistochemical staining was performed using the streptavidin-peroxidase method as described previously and 4-μm sections prepared as described above [12, 13]. After the sections had been deparaffinized and rehydrated in a descending series of alcohol dilutions, they were heated in an 800-W microwave oven at maximum power for 5 min in 0.01 mol/l citrate buffer (pH 6.0) for antigen retrieval, and cooled to room temperature. After blocking with goat serum (1:10 dilution, Invitrogen, Carlsbad, CA, USA) and incubated for 10 min at room temperature, the sections were incubated with primary mouse antihuman antibodies to BSP (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or OPN (1:500 dilution; Santa Cruz Biotechnology) for 20 min at room temperature. The slides were then washed three times using 0.1 M phosphate-buffered saline (PBS; pH 7.4) and incubated with a biotinylated horseradish peroxidase goat antimouse secondary antibody (1:500 dilution; Invitrogen) for 20 min at 37°C. The slides were washed three times after incubation with the secondary antibody using 0.1 M PBS (pH 7.4). Immunolabelling was visualized with 0.05% diaminobenzidine (Invitrogen) in 0.01 M PBS (pH 7.4) for 5 min at room temperature, and the slides were then rinsed for 2 min under running tap water. The immunohistochemical staining was examined and photographed at ×100 and ×400 magnification using an Olympus BX51 light microscope (Olympus Optical, Tokyo, Japan). The percentage of positive cells was evaluated semiquantitatively by counting the number of labelled cells in 10 randomly selected high-power fields for each specimen at ×400 magnification. Beast cancer specimens were provided by the Department of Pathology, Huashan Hospital to act as positive control samples for BSP and OPN immunostaining. For negative control slides, the primary antibody

Figure 1. Immunohistochemical staining showing strong OPN (A) or BSP (B) staining in PTC specimens compared to negative of OPN (C) or BSP (D) in benign thyroid nodules (A & C ×100 and C & D ×200).
Calcification and BSP/OPN in PTC

Table 2. IHS of OPN protein staining of PTC (n = 66)

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunohistochemical score</th>
<th>Positive n (%)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PTC tissue</td>
<td>66</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>Benign nodules</td>
<td>40</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>40</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Histological calcification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with calcification</td>
<td>25</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>without calcification</td>
<td>41</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Calcification on B-ultrasound</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with calcification</td>
<td>43</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>without calcification</td>
<td>23</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>LNM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>35</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Negative</td>
<td>31</td>
<td>5</td>
<td>17</td>
</tr>
</tbody>
</table>

Data presented as the number of specimens with the following immunohistochemical score: - negative; + weakly positive; ++ moderately positive; +++ strongly positive. PTC tissue versus benign nodules, Z = -2.363; two-tailed two-independent samples test. *PTC tissue versus normal tissue, Z = -4.389; two-tailed two-independent samples test.

Calcification was replaced with 0.1 M PBS (pH 7.4). Calcification was recognised by gross specimen and under microscopy.

An immunohistochemical score (IHS) was calculated for BSP and OPN for each specimen that took into account the percentage of cells (0-100%) as well as the staining intensity category (- to ++). The scoring system used for both BSP and OPN proteins was similar to previously published methods [14, 15]. The extent of positively stained cells was estimated and classified on a five-point scale as follows: grade 0, < 10%; grade 1, ≥ 10% and ≤ 25%; grade 2, > 25% and ≤ 50%; grade 3, > 50% and ≤ 75%; grade 4, > 75%. The intensity of the positive staining was categorized into three groups: weak (1); moderate (2); and strong (3). A final IHS score was obtained by multiplying the score for the extent and the score for intensity as follows: 0, negative (-); 1-4, weakly positive (+); 5-8, moderately positive (++); 9-12, strongly positive (+++).

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 11.5 (SPSS Inc., Chicago, IL, USA) for Windows®. Two-independent samples test or χ²-test was used to determine the difference between groups. Pearson’s contingency coefficient (C) was used to test for any associations between the levels of BSP and OPN proteins. A P-value of < 0.05 was considered statistically significant for all tests.

Results

Surgical resection specimens from 66 patients with PTC were retrieved from the archive of the Department of Pathology, Huashan Hospital. The surgical modalities that were used to treat the patients were as follows: PTC radical surgery + central lymph node clearance (level VI) (n = 55 with LNM); and PTC radical surgery + lateral cervical lymph node clearance (levels II-VI) (n = 35 with LNM; n = 3 cases without LNM). Immunohistochemical staining for BSP and OP proteins was undertaken on 35 patients with PTC with lateral cervical LNM and 31 patients with PTC without cervical LNM. A total of 40 benign thyroid nodules and 40 adjacent normal follicular epithelium specimens were collected for comparison.

The 66 patients with PTC (19 males and 47 females) had a mean age of 43.6 years (range 26-73 years). The 35 patients with PTC with lateral cervical LNM (14 males and 21 females) had a mean age of 42.8 years (range 26-61 years) and the 31 patients with PTC without cervical LNM (5 males and 26 females) had a mean age of 44.5 years (range 26-73 years). The 40 patients with benign thyroid nodules (11 males and 29 females) had a mean age of 43.4 years (range 21-69 years).

Immunohistochemical staining for BSP protein was performed on surgical specimens from 66 patients with PTC, 40 benign thyroid nodules, and 40 adjacent normal follicular epithelium specimens (Table 1; Figure 1). The percentage...
Table 3. Pearson's contingency coefficient analysis of the association between the level of immunohistochemical staining of BSP and OPN proteins in PTC (n = 66)

<table>
<thead>
<tr>
<th>OPN</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>52</td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>5</td>
<td>11</td>
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<tr>
<td>Total</td>
<td>58</td>
<td>8</td>
<td>66</td>
</tr>
</tbody>
</table>

Data presented as number of patients. $\chi^2$-test = 13.769; $P = 0.001$; Pearson's contingency coefficient (C) = 0.86.

Of samples that had cells that demonstrated moderate (++) to strong (+++) BSP staining in PTC specimens, benign thyroid nodules, and normal thyroid tissues was 68.18% (45/66), 10.00% (4/40), and 5.00% (2/40), respectively. The IHS for BSP protein staining was significantly higher in PTC specimens than in benign thyroid nodules and adjacent normal thyroid tissues ($P < 0.001$ for both comparisons). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 87.88%, 57.50%, 77.33%, and 74.19%, respectively.

Immunohistochemical staining for OPN was performed on specimens from 66 patients with PTC, 40 benign thyroid nodules, and 40 adjacent normal follicular epithelium specimens (Table 2; Figure 1). The percentage of samples that had cells that demonstrated moderate (++) to strong (+++) OPN staining in PTC specimens, benign thyroid nodules, and normal thyroid tissues were 50.00% (33/66), 17.50% (7/40), and 5.00% (2/40), respectively. The IHS for OPN protein staining was significantly higher in PTC specimens than in benign thyroid nodules and adjacent normal thyroid tissues ($P < 0.001$ and $P < 0.05$, respectively). The sensitivity, specificity, PPV, and NPV were 83.33%, 50.00%, 77.33%, and 64.52%, respectively.

Although there was not a significant difference in the IHS for both BSP and OPN protein staining between PTC specimens from patients with calcification and those who without calcification observed on B-ultrasound ($P > 0.05$), there was a significant difference in the IHS for both BSP and PN protein staining between PTC specimens from patients with histological calcification seen in paraffin sections and those who without histological calcification seen in paraffin sections. ($P < 0.05$ (Tables 1 and 2)).

Discussion

PTC is the most frequently observed malignant thyroid tumour (MTT), accounting for approximately 94.8% of all MTT cases [16]. The presence of calcification is the most significant ultrasonographic finding in evaluating thyroid nodules, calcifications are more frequently detected in PTC than in other thyroid lesions [5, 6, 17]. Histologically, calcification was classified as either psammoma bodies, stromal calcification, or bone formation, they were identified in 25%, 47%, and 13% of all the papillary thyroid carcinoma, respectively [18]. It is not fully understood why thyroid cancer is often complicated by calcification, and in particular microcalcification.

BSP and OPN are prominent, mineral-associated proteins in the extracellular matrix of bone that have been implicated in the metastatic activity of cancer cells [19]. BSP is a mineralized tissue-specific noncollagenous protein that is glycosylated, phosphorylated and sulfated [20], it plays an important role in cancer cell growth, migration and invasion [21, 22]. BSP expressed in most lung, breast, pancreatic cancer and multiple myeloma [8, 22, 23]. BSP can be detected in a variety of human cancers, particularly those that metastasize preferentially to the skeleton [8, 19, 24-26]. Although true follicular thyroid carcinoma is known to metastasize via the bloodstream and give rise to bone and lung metastases, such a pattern of spread is rare in papillary thyroid carcinoma [27]. Incidence of bone metastasis from follicular thyroid cancer was 6.8% (9 of 132 patients), and 0.4% (13 of 3,154 patients) from PTC [28]. But most of the thyroid carcinomas (72%) examined expressed high levels of BSP, among the differentiated tumors, PTCs exhibited the
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highest level of BSP expression when compare with follicular thyroid carcinomas and medullary thyroid carcinomas [8].

Karadag et al., suggested that BSP interacts separately with both matrix metalloproteinase 2 (MMP2) and integrin alpha (v) beta (3) and is overexpressed in many metastatic tumors [29]. BSP enhances cancer cell invasiveness by forming a trimolecular complex with MMP2 and cell-surface integrin alpha (v) beta. We previously reported that there was a significant difference in the immunohistochemical score for MMP2 protein staining between PTC specimens from patients who developed lateral cervical LNM and those who did not develop LNM [30]. From this study, we found that BSP protein staining is positive in 87.88% patients, BSP was significantly higher in PTC specimens than in benign thyroid nodules and adjacent normal thyroid tissues. But there was not a significant difference in the IHS for BSP protein staining between PTC specimens from patients who developed lateral cervical LNM and those who did not develop LNM (P > 0.05). We suggest an important role for BSP in calcification and tumor development in PTC.

OPN is an integrin-binding protein, believed to be involved in a variety of physiological cellular functions. It has been shown to play an important role in tumorigenesis, tumor invasion, and metastasis in breast, lung, prostate, colon, ameloblastomas, and gastric cancers [9, 10, 31, 32]. OPN mRNA was significantly overexpressed in the PTC samples compared with other thyroid tumors [33]. Guarino et al., reported that the prevalence and intensity of OPN staining were significantly correlated with the presence of LNM and tumor size in human PTCs [34]. OPN protein staining was positive in 81.97% patients in this study, its staining was significantly higher in PTC specimens than in benign thyroid nodules and adjacent normal thyroid tissues (P < 0.05 and P < 0.001, respectively). But there was not a significant difference in the IHS for OPN protein staining between PTC specimens from patients who developed lateral cervical LNM and those who did not develop LNM (P > 0.05).

We previously reported that there was a significant difference in the immunohistochemical score for CD44v6 protein staining between PTC specimens from patients who developed lateral cervical LNM and those who did not develop LNM [30]. Guarino et al., suggested that OPN is able to induce CD44v6 overexpression, it is conceivable that in PTC cells, the RET-RAS-BRAF-MAPK oncogenic cascade triggers OPN and CD44v6 up-regulation; this leads to OPN-CD44v6 binding, thereby further increasing CD44v6 up-regulation and enhancing MAPK and AKT signaling [34].

Microcalcifications are often associated with human mammary lesions, particularly with breast carcinomas. Expression of BSP by cancer cells could play a major role in the mineral deposition and in preferred bone homing of breast cancer cells [20]. When microcalcifications were observed in pulmonary malignant lesions, they were usually associated with cancer cells expressing BSP [23].

There is a close association between microcalcification and thyroid cancer. Psammoma bodies (PBs) which are concentric lamellated calcified structures are mainly related to microcalcification [35], which supports the idea that calcification may be linked with the development of malignant tumours and confirms the close association between microcalcification and thyroid cancer. The presence of OPN, which is produced by macrophages, has been shown to be associated with the development of PBs in PTC [36], further studies on OPN also support the theory of calcification due to tumour development and progression [18]. OPN mRNA-expressing cells were present around the PBs, and the localization of OPN protein was consistent with that of PBs [36]. PBs are said to represent a process of dystrophic calcification. Ultrastructural study of PTC has shown that thickening of the base lamina in vascular stalk of neoplastic papillae followed by thrombosis, calcification, and tumor cell necrosis leads to formation of PBs [37]. Das et al., suggested that rather than being the outcome of dystrophic calcification of dead or dying tissue, PBs may indeed represent an active biologic process ultimately leading to degeneration/death of tumor cells and retardation of growth of the neoplasm [37].

From our study, calcification on B-ultrasound was seen in 43 patients with malignant nodules and it was confirmed on paraffin sections in 25 patients. Although there was not a signifi-
cant difference in the IHS for both BSP and OPN protein staining between PTC specimens from patients with calcification and those who without calcification observed on B-ultrasound (P > 0.05), there was a significant difference in the IHS for both BSP and OPN protein staining between PTC specimens from patients with histological calcification seen in paraffin sections and those who without histological calcification seen in paraffin sections. The level of BSP protein staining was found to be significantly correlated with the level of OPN protein staining in PTC specimens. The prognostic value of BSP and OPN detection in PTC and the potential role of BSP and OPN in the propensity of this type of cancer to metastasize to bone are under investigation. Bai et al., suggested that PTC with, compared to that without, psammoma bodies was associated with poorer disease-free survival, they suggest that the presence of psammoma bodies is a useful predictor of outcome for patients suffering from PTC [18].

In conclusion, the strong correlation between BSP and OPN and PTC suggests a role for BSP and OPN in calcification and tumor development and progression, BSP and OPN might be useful tumour markers for the diagnosis of PTC with limited value, because both of them had very low specificity. This study was a retrospective study with a small sample size, so these data will need to be confirmed in larger, prospective, multicentre studies in the future.

Disclosure of conflict of interest

None.

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References

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