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Original article

Papillary thyroid carcinoma with bone formation

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ABSTRACT

Bone formation is a rarely encountered finding during histological examination of papillary thyroid carcinoma (PTC). This study aimed to analyze clinicopathological parameters in patients with PTC showing bone formation, to document histological features of bone formation in PTC, and to investigate osteogenic proteins. Bone morphogenic protein (BMP)-9 is known as the most potent osteoinductive protein of the BMP subtypes. Recent research suggests that the activin receptor-like kinase (ALK) 1 is an essential cellular receptor that mediates BMP-9-induced osteogenic signaling. A retrospective review of tumor sections from 567 patients with a diagnosis of PTC was performed. Using immunohistochemistry and quantitative real-time polymerase chain reaction, we investigated the expression of ALK1 and BMP-9 in normal thyroid tissue and PTC samples with and without bone formation. Bone formation was found in 13% of patients with PTC. A significant association was seen between bone formation and old age. BMP-9 expression in tumors was increased compared to that in normal thyroid tissues. BMP-9 expression in tumors with bone formation was not significantly different from that in tumors without bone formation. ALK1 expression in tumors with bone formation was increased compared to that in normal thyroid tissue and tumors without bone formation. Our study suggests that upregulation of ALK1 might be an underlying molecular mechanism that explains osteogenesis in PTC.

Introduction

Bone formation is a rarely encountered finding in the histological examination of papillary thyroid carcinoma (PTC). Two earlier studies have investigated bone formation in PTC [1,2]. However, little is known about the epidemiological and histological features, as well as the pathogenesis of bone formation in PTC. This study aimed to analyze the clinicopathological parameters of PTC patients with regard to bone formation, and to document the histological features of bone formation in PTC. We also investigated osteogenic proteins in an effort to explain the mechanism of osteogenesis in PTC.

Among the osteoinductive signaling proteins, the most important ones are bone morphogenic proteins (BMPs), which belong to the transforming growth factor-beta (TGF-β) superfamily. BMPs play a critical role in heart, neural, and cartilage formation during postnatal bone formation. Genetic mutations in BMPs can cause skeletal and extraskeletal defects [3]. More than 15 types of BMPs have been identified in humans, and BMP-2, -4, and -7 are closely associated with osteogenesis. In addition, BMP-9 was found to be the most potent BMP subtype for inducing osteogenic differentiation [4].

BMPs initiate signaling by binding to type I or type II BMP receptors, which are transmembrane serine/threonine kinases. BMP and BMP receptors form quaternary complexes and activate the Smad pathway, which mediates extracellular signaling into the nucleus and triggers gene expression. Smad proteins transactivate osteoblastogenic genes either directly or through the early and late osteoblast transcription factors Runx2 and osterix [5,6].

Activin receptor-like kinase (ALK) 1 is one of seven type I BMP receptors (ALK1-7). Recent studies have demonstrated coordination of BMP-9 and ALK1 in angiogenesis, proliferation of endothelial cells, and differentiation of vascular mesenchymal stem cells [7,8]. In addition, ALK1 has been implicated as an essential molecule in BMP-9-induced osteogenesis [9,10].

Using immunohistochemistry and quantitative real-time polymerase chain reaction (qPCR), we investigated whether BMP-9 and ALK1 expressions were changed in PTC with bone formation.

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Materials and methods

Patients and tissue samples

We retrospectively reviewed the clinical records of patients with a diagnosis of thyroid cancer who underwent surgical treatment at Hyung Hee University Hospital from 2010 to 2011. We found 575 patients and excluded 5 with follicular carcinoma, 2 with medullary carcinoma, and 1 with anaplastic carcinoma, enrolling 567 patients with PTC. All tumor sections stained with hematoxylin and eosin from the 567 cases of PTC were available. Two pathologists (K.Y.N. and Y.K.P.) reviewed all hematoxylin and eosin-stained slides for the presence of bone formation and analyzed histological features of bone formation. Representative formalin-fixed, paraffin-embedded tissue samples of 76 cases of PTC with bone formation and 64 cases of PTC without bone formation were used for immunohistochemistry. Representative formalin-fixed, paraffin-embedded tissue samples of 15 normal thyroid tissue, 15 cases of PTC with bone formation, and 15 cases of PTC without bone formation were used for qPCR.

RNA isolation

Total RNA was isolated from formalin-fixed, paraffin-embedded tissues using RNeasy kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To accommodate more tissue, the protocol was modified so that all slices were placed in a 1.5 ml tube and deparaffinized together (two washes with 1 ml 100% xylene followed by two washes with 1 ml 100% ethanol followed by drying in a Speedvac for 10 min). Dried tissue was resuspended in 500 μl proteinase K buffer, and the remaining steps were performed as per the manufacturer's protocol.

Quantitative real-time polymerase chain reaction

Total RNA (500 ng) was reverse transcribed with SuperScript II (Invitrogen, Carlsbad, CA, USA) using an oligo-dT primer, as described by the manufacturer. An amplification reaction was carried out in 20 μl with 1.0 μl cDNA, 20 pM of forward and reverse gene-specific primers, specific TaqMan probes from the Universal Probe Library (Roche Diagnostics GmbH, Mannheim, Germany), and 2× LC480 Probes Master Mix (Roche) using a LightCycler 480 system. Primers for qPCR were: ALK1-F: 5′-CGC AAT GTG CTG GTC AAG-3′; ALK1-R: 5′-GTT GCC GAT GTC CAG GTA AT-3′; BMP-9-F: 5′-GGG CAC AAC AAG GCA TGA TT-3′; BMP-9-R: 5′-AGA GCA GGC TCC CTT TCC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of RNA integrity. Changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry

We used the Bond Polymer Intense Detection System (Vision Biosystems, Mt Waverley, VIC, Australia) according to the manufacturer's instructions with minor modifications for immunohistochemistry. Sections (4 μm) from tumor specimens were deparaffinized with Bond Dewax Solution, and antigen retrieval was performed using Bond ER1 (pH 6.0) solution for 30 min at 100 °C. Endogenous peroxidases were quenched by incubation with hydrogen peroxide for 5 min. Sections were incubated for 15 min with primary antibodies at ambient temperature using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system in a Bond-Max autostainer. Primary antibodies were rabbit polyclonal BMP-9 antibody (Abcam, UK, 1:200) and rabbit polyclonal ALK1 antibody (Abcam, 1:200). Nuclei were counterstained with hematoxylin. Normal human lung tissue was used as a positive control. The negative control was normal adjacent tissue processed without primary antibody.

Assessment of immunoreactivity

The immunohistochemical evaluation was performed by two pathologists (K.Y.N. and Y.K.P.) who were blinded to the clinicopathological features. More than 70% of cells in both tumor and normal follicles showed homogeneous immunohistochemical signals in each sample, irrespective of intensity. Therefore, immunoreactivity was scored by staining intensity: no visible brown staining, 0; pale tan to brown, 1+; homogeneous coffee color, 2+; dark brown granular, 3+.

Statistics

Statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL, USA). Relative levels of mRNA are shown as mean with standard deviation. Differences between mean values were analyzed using independent *t*-tests. A chi-square test or Fisher's exact test was performed to analyze the relationship of the clinicopathological parameters, bone formation, and antibody expression. Logistic regression analysis was used to determine the parameters that correlated with the bone formation using the backward stepwise elimination method. A P-value < 0.05 was considered significant.

Results

Correlation of bone formation and other clinicopathological parameters in papillary thyroid carcinoma

Bone formations were present in 78 of 567 (13%) cases of PTC. The presence of bone formation correlated with older age ($P = 0.027$), extrathyroid invasion ($P = 0.008$), pT ($P = 0.037$), and stage grouping ($P = 0.008$). The pT and stage groupings were classified according to the TNM classification of American Joint Committee on Cancer. No association was seen between the presence of bone formation and sex, tumor size, the presence of lymphocytic thyroiditis, lymph node metastasis, or preoperative serum calcium or phosphate level (Table 1). In logistic regression analysis, only patient age was associated with the presence of bone formation ($P = 0.003$).

Histological features of the bone formation in papillary thyroid carcinoma

A dense fibroblastic stroma was observed in 76 PTC samples with bone formation; 74 of the 76 (97%) samples showed stromal calcification. Ossification sites were edge of stromal calcification (60 of 76, 79%), fibroblastic stroma without calcification (11 of 76, 14%), and intratumoral areas (5 of 76, 6%) (Fig. 1). Ossification size ranged from <0.1 cm to 0.8 cm (median, 0.2 cm). Osteocytes and the osteoid matrix were constitutively identified, whereas osteoblastic rimming, bone marrow, and the lamellated bone matrix were detected only when the ossification size was >0.3 cm. Of 76 tumor samples, 16 showed bone marrow; 3 of the 16 showed hematopoietic marrow, and 13 showed fatty marrow (Table 2).

ALK1 and BMP-9 immunoreactivity in papillary thyroid carcinoma

ALK1 and BMP-9 were cytoplasmically localized in tumor cells and normal follicular cells with variable intensity (Fig. 2). No immunoreactivity was seen for ALK1 and BMP-9 in spindle cells in fibroblastic stroma, osteocytes, or osteoblast. Table 3 summarizes