RESEARCH ARTICLE

Osteopontin Levels in Patients with Papillary Thyroid Cancer According to the Presence of Hashimoto's Thyroiditis

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Abstract

Background: Human papillary thyroid carcinoma (PTC) is often associated with Hashimoto's thyroiditis (HT); their coexistence improves PTC prognosis. Osteopontin, a secreted glycoprotein, plays a role in cell survival, immunity, and tumor progression, its expression being associated with a poor prognosis and metastasis in several malignancies. Osteopontin overexpression correlates with aggressive clinicopathological features in PTC. Lymph node metastases and large tumor size positively correlate with osteopontin positivity. This study aimed to: (1) confirm osteopontin overexpression in human PTC samples; (2) compare osteopontin expression levels in PTC cases with and without HT; and (3) identify correlations between tumor aggressiveness and osteopontin expression levels. Materials and Methods: Plasma osteopontin was assessed in 45 patients with PTC, 22 patients with PTC and HT, and 24 healthy controls by enzyme-linked immunosorbent assay. Thyroid tissue osteopontin mRNA and protein levels were analyzed by reverse transcription-polymerase chain reaction and Western blotting, respectively. Results: Plasma osteopontin levels were significantly higher in PTC patients than in healthy controls. Plasma osteopontin, tissue osteopontin mRNA, and tissue osteopontin protein levels were significantly lower in patients with PTC and HT than in those with PTC alone. In advanced disease stage cases, osteopontin mRNA and protein expression levels were lower in patients with PTC and HT than in those with PTC alone. However, the osteopontin expression level was not significantly associated with the TNM stage. Conclusions: Plasma osteopontin, tissue osteopontin mRNA, and tissue osteopontin protein levels were significantly lower in patients with PTC and HT than in those with PTC alone, suggesting that HT attenuates PTC aggressiveness through negative regulation of osteopontin expression.

Keywords: Osteopontin - papillary thyroid cancer - Hashimoto's thyroiditis

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Introduction

Thyroid cancer is the most common malignancy of the endocrine system. Papillary thyroid carcinoma (PTC), the most prevalent manifestation of thyroid cancer, has a 10-year relative survival rate of 93% (Hundahl et al., 1998). In Korea, the prevalence of PTC is rapidly increasing, accounting for >95% of all cases of thyroid cancer (Kwak et al., 2010). Hashimoto's thyroiditis (HT) is a chronic inflammation of the thyroid gland and the most common cause of hypothyroidism in iodine-sufficient areas of the world, with a particularly high prevalence among older individuals (Hollowell et al., 2010). The association between HT and PTC was first described by Dailey et al. in 1955 (Dailey et al., 1955). Although HT is known to increase the risk of PTC (Fiore et al., 2011; Kim et al., 2011). the co-presence of HT and PTC is associated with a less aggressive clinical presentation and low recurrence rate (Kim et al., 2009; Huang et al., 2011). Differentiated thyroid cancer, including PTC, usually expresses thyroid specific antigens, similar to those expressed by normal follicular epithelial cells. In this regard, the autoimmune response to thyroid-specific antigens in HT may target destruction of cancer cells that also express thyroid-specific antigens in PTC patients, thus preventing recurrence and improving survival. However, the association between the two diseases has remained under debate.

Osteopontin (OPN) is a secreted glycoprotein that has been extensively studied for its involvement in osteoblast differentiation and bone formation (Giachelli et al., 2000). OPN has multifunctional properties for cell migration and cell survival. It is expressed in several types of cancer and has been associated with tumorigenesis and invasion. Tumor cells with high metastatic potential express high levels of OPN (Oates et al., 1999) and elevated plasma

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OPN levels are associated with poor patient survival (Fedarko et al., 2001). In a recent study, OPN was found to be overexpressed in human PTCs, and the prevalence and intensity of OPN correlated with aggressive features (Guarino et al., 2005). Here, we confirmed the overexpression of OPN in PTC patients, compared the expression of OPN in PTC patients with and without HT, and assessed the correlation between OPN expression and aggressiveness in patients with PTC.

Materials and Methods

Participants

Between April 2008 and July 2009, 103 consecutive patients were referred and screened for this study. We excluded patients with other cancers and autoimmune disorder, diabetes mellitus, renal disorder, liver disorder, or any other inflammatory or medical condition that would have influenced the parameters under study. After all exclusions, a total of 91 patients were enrolled in our present study, consisting of 13 men and 78 women with a mean age of 46 years.

Of the 91 patients, 24 were healthy controls, 45 had PTC alone, and 22 had PTC with HT. Tissue samples were obtained during thyroidectomies performed at the Department of Surgery at Ulsan University Hospital. After surgical resection, thyroid samples were prepared by a pathologist who selected tumor and normal tissues. Thyroid tissue samples were frozen in liquid nitrogen and stored at -70°C. Blood was collected from patients before surgery and from healthy volunteers as the normal controls. Tumor staging was based on the recommendations of the seventh edition of the American Joint Committee on Cancer.

Enzyme-linked immunosorbent assay

Plasma samples obtained from patients and healthy controls and stored at -70°C were used for quantification of the OPN level by enzyme-linked immunosorbent assay (ELISA) (Duo Set ELISA kit, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 100 μL of assay diluent was added to each well, followed by the addition of standard, control, or plasma samples and incubation for 2 h at room temperature. After aspiration from each well and four washes with wash buffer, 200 μL of conjugate was added to each well, incubated for 2 h, and the last washing step was repeated. Substrate solution was then added to each well and incubated for 30 min at room temperature. The reaction was stopped and absorbance was read on an ELISA plate reader at 450 nm. The level of OPN was calculated according to a standard curve.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from PTC tissue samples was prepared using the TRI reagent (Sigma Aldrich, St Louis, MO, USA).

Approximately 1 μg of total RNA was converted into cDNA using the AccuScriptTM high fidelity first strand cDNA synthesis kit (Stratagene, Santa Clara, CA, USA) at 65°C for 5 min, 42°C for 1 h, and 70°C for 15 min. For the PCR reaction, 10× buffer, 10 Mm dNTP mix, Taq polymerase (New England Biolabs, Ipswich, MA, USA), and specific primers were mixed with cDNA. The following primers were used for the amplification of OPN and β-actin:

OPN forward, 5'-GCCGAGGTGATAGTGTGGTT-3' and reverse, 5'-TGAGGTGATGTCCTCGTCTG-3'; β-actin forward, 5'-AAATCTGGCACCACACCTTC-3' and reverse, 5'-TGATCTGGGTCATCTTCTCG-3'.

PCR products were subjected to 1.5% agarose gel electrophoresis and visualized under UV light. Images were acquired using the Bio-Rad Molecular Imager ChemiDoc XRS (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Total protein extracts were prepared using the T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) containing a cocktail of protease inhibitors (Pierce, Rockford, IL, USA). Protein levels were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Proteins were separated by SDS-PAGE on 9–12% polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore Corp, Bedford, MA, USA). Membranes were blocked with Tris-buffered saline/0.1% Tween 20 (TBST) containing 5% nonfat dry milk for 60 min at room temperature. After washing four times with TBST, membranes were incubated with primary antibodies at 4°C overnight, washed four times with TBST, and incubated with horseradish peroxidase-conjugated species-specific secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature and then washed again four times. Signals were visualized using the Bio-Rad Molecular Imager ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) with the Immun-Star™ WesternC™ Chemiluminescent kit (Bio-Rad, Hercules, CA, USA).

Statistical analysis

One- and two-way analyses of variance with post-hoc analysis using Tukey's test (GraphPad ver. 5, Prism) and Student t-tests were used to evaluate the significance of the results. P values <0.05 were considered statistically significant. Data are expressed as mean±standard deviation.

Results

Plasma osteopontin levels were higher in papillary thyroid cancer patients than in healthy controls

Analysis of plasma OPN levels in 67 PTC patients and 24 healthy controls showed significantly higher OPN levels in PTC patients than in the controls (mean plasma OPN level: 66.1 ng/mL *vs* 36.9 ng/mL, p<0.001)

Patients with PTC were divided into two groups: those with PTC alone (n=45) and those with PTC and HT (n=22).

Significant differences in plasma OPN levels were found between the healthy controls and the patients with PTC alone (p<0.001) and between patients with PTC and HT patients and those with PTC alone (p<0.001).

However, no significant differences in plasma OPN levels were detected between healthy controls and patients with PTC and HT (mean plasma OPN level: 36.9 ng/mL for controls, 72.6 ng/mL for patients with PTC alone, and 53.0 ng/mL for patients with PTC and HT; shown in Figure 1).

Tissue OPN mRNA expression was lower in patients with PTC and HT than in those with PTC alone

Tissue OPN mRNA expression levels were significantly lower in patients with PTC and HT than in those with PTC alone ($p<0.05$), and the mean tissue OPN mRNA levels were 1.48-fold and 3.32-fold higher, respectively, in these groups than those of healthy controls (Figure 2).

Tissue OPN protein expression was lower in patients with PTC and HT than in those with PTC alone

Tissue OPN protein expression levels were significantly lower in patients with PTC and HT than in those with PTC alone ($p<0.05$), and the mean tissue OPN protein levels were 1.28-fold and 2.17-fold higher, respectively, in these groups than those of healthy controls (Figure 3).

Expression of OPN according to T stage in patients with PTC alone and in those with PTC and HT

To investigate the possible correlation between the OPN expression level and tumor aggressiveness, the plasma OPN, tissue OPN mRNA, and tissue OPN protein levels were compared according to T stage (T1-T2, T3-T4) and the presence of lymph node metastasis (N0, N1). OPN expression did not differ significantly between patients with early and those with advanced stages of disease among patients with PTC and HT. However, tissue OPN protein levels were significantly higher in T3-T4 cases than in T1-T2 cases among patients with PTC alone ($p=0.032$); plasma OPN and tissue OPN mRNA levels were also higher in T3-T4 patients, but the difference was not statistically significant.

Mean plasma OPN levels in cases of T1-T2 and T3-T4 stages were 66.1 and 75.9 ng/mL, respectively, in patients with PTC alone and 69.7 and 61.2 ng/mL, respectively, in patients with PTC and HT (Figure 4A). Mean relative tissue OPN mRNA expression in cases of T1-T2 and T3-T4 stages was 2.68 and 3.79, respectively, in patients with

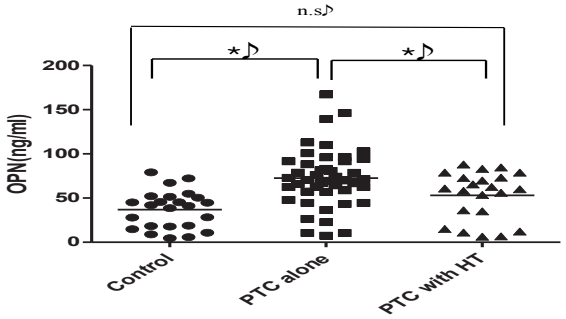

Figure 1. Plasma Opstepontin (OPN) Levels in Healthy Controls (n=24) and Papillary Thyroid Cancer (PTC) Alone (n=45), PTC with HT(Hashimoto's thyroiditis) Patients (n=22). OPN levels in plasma were measured by enzyme-linked immunosorbent assay. Mean value for each group is shown by a horizontal bar. *p<0.001, n.s: no significant

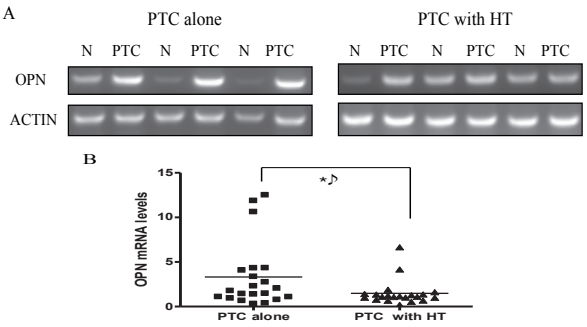

Figure 2. OPN mRNA Expression in PTC Alone and PTC with HT. (A) RT-PCR analysis of OPN mRNAs levels on normal thyroid tissue and cancer tissue. **(B)** Representation of densitometric analysis of OPN expression relative to normal levels derived from experiments. *p<0.005

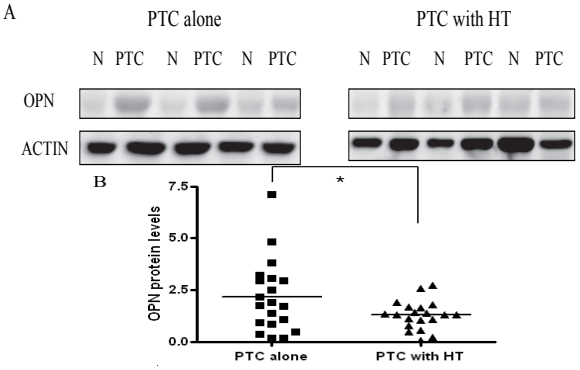

Figure 3. OPN Protein Expression in PTC Alone and PTC with HT. (A) Western blot analysis of OPN levels on normal thyroid tissue and cancer tissue. **(B)** Representation of densitometric analysis of OPN expression relative to normal levels derived from experiments. *p<0.005

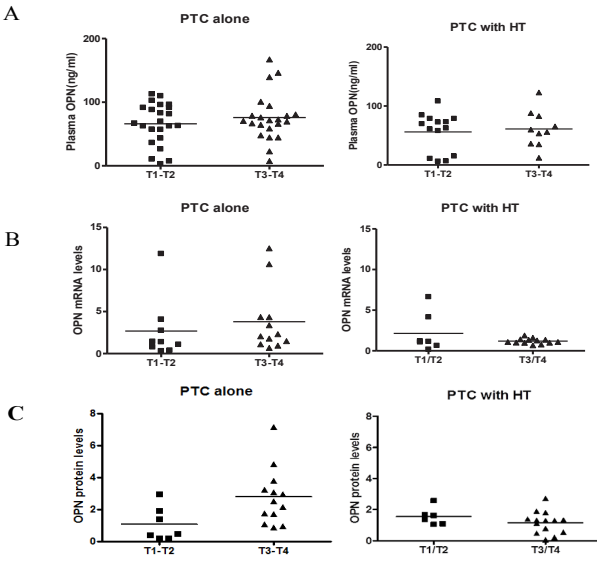

Figure 4. Expression of OPN in Relation to T Stage of PTC Alone and PTC with HT. (A) plasma OPN: Mean value was 66.1ng/ml in T1/T2 and 75.9ng/ml in T3/T4 PTC alone patient($p=0.352$), 69.7ng/ml in T1/T2 and 61.2ng/ml in T3/T4 PTC with HT patient ($p=0.6899$). **(B)** OPN mRNA; Mean value was 2.68 in T1/T2 and 3.79 in T3/T4 PTC alone patient($p=0.5121$), 2.15 in T1/T2 and 1.19 in T3/T4 PTC with HT($p=0.1584$). **(C)** OPN protein; Mean value was 1.06 in T1/T2 and 2.77 in T3/T4 PTC alone patient($p=0.0318$), 1.56 in T1/T2 and 1.16 in T3/T4 PTC with HT ($p=0.2393$)

PTC alone, and 2.15 and 1.19, respectively, in those with PTC and HT (Figure 4B). In advanced cases, tissue OPN mRNA expression was significantly higher in patients with PTC alone than in those with PTC and HT (p=0.023). Mean tissue OPN protein expression in T1-T2 and T3-T4 stages was 1.06 and 2.77, respectively, in patients with PTC alone, and 1.56 and 1.16, respectively, in those with PTC and HT (Figure 4C). In advanced stages, tissue OPN protein expression was significantly higher in patients with PTC alone than in those with PTC and HT (p=0.043).

The presence of lymph node metastasis and TNM stage were not associated with plasma OPN levels and tissue OPN mRNA and OPN protein levels.

Discussion

The relationship between PTC and HT remains unclear because there is no conclusive evidence as to whether HT is secondarily induced by the neoplasm or HT predisposes the patient to develop thyroid cancer. In previous studies, the coexistence of PTC and HT was found to be associated with smaller tumor size, younger age, female predominance, reduced risk of recurrence, lower incidence of extranodal extension and a lower lymph node metastasis ratio, early stage disease, and better prognosis compared to PTC alone (Kim et al. 2009; Kim et al. 2011; Ahn et al., 2011; Loh et al., 1999; Singh et al., 1999; Kashima et al., 1998). Although autoimmune mechanisms have been suggested to explain the better prognosis of patients with PTC and HT compared to those with PTC alone, the underlying mechanism remains unclear.

OPN is a glycosylated phosphoprotein that has been associated with many physiological and pathological processes including wound healing, bone formation, tumorigenesis, inflammation, ischemia, and immune responses (Wang et al., 2008). The role of OPN in tumorigenesis, cancer progression, and survival has been demonstrated in various cancers (Rittling et al., 2004) and OPN has been suggested to contribute to tumor progression by enhancing angiogenesis through a vascular endothelial growth factor (VEGF)-dependent mechanism, the so-called OPN-VEGF axis (Liu et al., 2011).

Guarino et al. showed that the presence of lymph node metastases and large tumor size both positively correlated with OPN positivity in an immunohistochemical study of human thyroid tumor samples (Guarino et al., 2005). In another study, the authors, using RT-PCR of fresh-frozen tissue of thyroid tumors, found that PTC has higher OPN expression than other thyroid tumors and lymph node metastasis (Kung et al., 2013). Furthermore, Briese et al. showed that OPN is expressed in benign and malignant thyroid tumors, with increasing expression correlating with advancing malignancy (Briese et al., 2010). Despite this pattern of expression, the authors were unable to show the value of OPN as a prognostic marker, some of which are tumor size, multifocal growth, extrathyroidal extension, vascular invasion, lymph node metastasis, and distant metastasis as well as gender and age for patients with malignant thyroid tumors. In this study, we investigated the correlation between OPN and tumor aggressiveness in patients with concomitant PTC and HT by comparing the expression level of OPN in the plasma and tissues of patients with PTC alone with those in patients with PTC and HT. Similar to the study discussed above, we confirmed higher OPN expression in PTC patients than in healthy controls, but, interestingly, plasma OPN, tissue OPN protein, and OPN mRNA levels were significantly lower in patients with PTC and HT patients than in those with PTC alone. As far as we know, differences in OPN expression levels between patients with PTC and HT and those with PTC alone have not been reported. In the present study, OPN expression levels in the overall cohort of PTC patients did not reflect tumor aggressiveness, likely because the entire patient cohort included patients with HT, who showed lower OPN levels. However, in patients with PTC alone, tissue OPN mRNA expression levels were significantly higher at advanced stages than at early stages. Moreover, OPN mRNA and OPN protein levels in patients with HT and PTC were significantly lower than those in patients with PTC alone, at advanced stages. These results suggest that the presence of concomitant HT may decrease the aggressiveness of PTC by negatively regulating OPN expression.

In conclusion, the results of this study indicate a significant difference in OPN expression levels between patients with PTC and HT and those with PTC alone. Therefore, when using OPN levels to diagnose PTC patients, the co-presence of HT should be considered. Further investigation is needed to elucidate the mechanisms underlying the relationship between HT and OPN expression.

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