

Polymorphisms in bone morphogenetic protein 3 and the risk of papillary thyroid cancer

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Abstract. Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGF β) superfamily with well-described functions in bone formation. Although disrupted BMP signaling in tumor development has been investigated, a genetic association for BMP3 in papillary thyroid cancer (PTC) has remained largely unexplored. In this study, we investigated whether BMP3 single nucleotide polymorphisms (SNPs) are associated with the development of PTC and its clinicopathological features. A total of 103 PTC patients and 324 control subjects were enrolled. One promoter SNP (rs13138132; -1919C/A) and one missense mutation (rs3733549; Arg192Gln) in BMP3 were genotyped by direct sequencing. SNPStats, SNPAnalyzer, HelixTree and Haploview version 4.2 were used to evaluate the genetic data. Multiple logistic regression models were used to calculate odds ratios (ORs), 95% confidence intervals (CIs) and P-values. The missense SNP (rs3733549) was weakly associated with the development of PTC in a codominant model (AA vs. GG; P=0.017) and a recessive model (AA vs. GG/GA; P=0.023). Additionally, in an analysis according to clinicopathological features, rs13138132 was significantly associated with extrathyroidal invasion in a codominant model (CA vs. CC; P=0.006) and a dominant model (CA/AA vs. CC; P=0.0023). We also identified that the frequency of the A allele in the promoter SNP (rs13138132) was increased in PTC patients

with extrathyroidal invasion (P=0.004). Our data suggest that rs3733549 in BMP3 is associated with the development of PTC and that the A allele of rs13138932 in BMP3 is a risk factor for extrathyroidal invasion.

Introduction

Thyroid cancer is the most common type of endocrine malignancy, accounting for approximately 1% of all cases of cancer. It is the most rapidly increasing cancer among females and the second most rapidly increasing cancer among males. Histologically, thyroid cancer is classified as papillary thyroid cancer (PTC), follicular thyroid cancer, medullary thyroid cancer or undifferentiated or anaplastic thyroid cancer. Of these, PTC is the most common type, and it accounts for 85-90% of all thyroid malignancies (1,2). Although the etiology of PTC is unclear, environmental factors, including radiation, diet, smoking and hormones, have been identified to affect the pathogenesis of thyroid cancer (3,4). Genetic predisposition has also been implicated as a risk factor for thyroid cancer development (5,6).

Bone morphogenic proteins (BMPs) have been demonstrated to play an important role during development (particularly during bone formation) and in the regulation of various cellular processes, including cell proliferation, apoptosis and differentiation (7). Evidence suggests that BMPs are important in tumorigenesis (8-10). BMPs are members of the transforming growth factor beta (TGF β) family; thus, BMPs utilize a similar signaling cascade to that of TGF β . BMPs have been identified to utilize the SMAD signaling pathway for their growth suppressive effects, and also to affect PTEN and p21 (WAF1) expression via RAS-ERK signaling in cancer (10-12). Additionally, previous studies have revealed that genetic variations in BMP genes are associated with a number of types of cancer (13-15). Genome-wide association studies have demonstrated associations between polymorphisms in BMP2 (13) and BMP4 (13,14) and colorectal cancer. Park *et al* (15) also

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suggested that a frame-shift mutation in BMP receptor type II (BMPRII) contributes to the pathogenesis of gastric and colorectal cancers by inactivating BMPRII-mediated BMP signaling.

BMP3 plays an important role in embryonic development by inducing and patterning early skeletal formation. *In vitro* studies have also suggested a role for BMP3 as a negative growth regulator in bone marrow progenitor cells through the inhibition of DNA synthesis and proliferation (16). Additionally, recent studies have revealed that BMP3 contributes to tumor development and progression (17-19). However, to the best of our knowledge, no study of a possible genetic association between polymorphisms in BMP3 and cancer has been conducted. In this study, we investigated the association between single nucleotide polymorphisms (SNPs) in BMP3 and PTC, as well as the clinicopathological features of PTC, including cancer size, tumor number, cancer location, extrathyroidal invasion and metastasis. To the best of our knowledge, this is the first study to investigate the association between BMP3 SNPs and cancer, with particular interest in PTC.

Materials and methods

Patients and control subjects. PTC patients were enrolled at Kyung Hee University Medical Center, Seoul, Korea, between October 2007 and December 2010. Control subjects were selected from healthy individuals examined under a general health check-up program to ensure that there was no clinical evidence of cancer, thyroid disease or any other severe conditions. PTC and cervical region lymph node metastasis were confirmed by pathological examination. Specimens confirmed as a benign tumor, follicular variant, diffuse sclerosing variant or tall cell variant were excluded. The mean age of the patients was 54.7±12.3 years. The patient group (n=105) was comprised of 29 males and 76 females, while the control group consisted of 324 healthy adults (53.8±6.0 years), including 132 males and 192 females. This study was approved by the ethics review board of the Medical Research Institute, Kyung Hee University Medical Center (KMC IRB 1010-05). Written informed consent was obtained from each subject prior to study entry.

Patient subgroups. To determine the nature of the correlation between BMP3 polymorphisms and the clinicopathological characteristics of PTC, the patients were divided into subgroups according to the size of the tumor (<1 vs. ≥1 cm), number of tumors (unifocal vs. multifocal), location of the cancer (one lobe vs. both lobes), extrathyroidal invasion (presence vs. absence), cervical lymph node metastasis (presence vs. absence) and angiolymphatic invasion (presence vs. absence). The demographic characteristics of the PTC patients are summarized in Table I; small differences in subgroup numbers were caused by the loss of certain clinical data.

SNP selection and genotyping. We searched for promoter and coding SNPs in BMP3 in the SNP database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP>; BUILD 135). Those SNPs without genotype

Table I. Clinical characteristics of the study population.

Characteristics	PTC (n)	Control
Age, years (mean ± SD)	54.7±12.3	56.3±11.9
Gender		
Male	29	132
Female	76	192
Tumor size, cm		
<1	53	
≥1	50	
Number of tumors		
Unifocal	66	
Multifocal	35	
Cancer location		
One lobe	65	
Both lobes	36	
Extrathyroidal invasion		
Absent	49	
Present	53	
Cervical lymph node metastasis		
Absent	67	
Present	30	
Angiolymphatic invasion		
Absent	94	
Present	6	

PTC patients with inappropriate clinical data were excluded. PTC, papillary thyroid cancer; SD, standard deviation.

frequency data and with a heterozygosity ≤0.1 or a minor allele frequency ≤0.1 were excluded. Ultimately, two SNPs (rs13138132, -1919C/A; rs3733549, Arg192Gln) were selected. Blood samples for DNA extraction were collected from each subject in ethylene diamine tetracetic acid (EDTA) tubes and stored at -80°C. Genomic DNA was extracted using a QIAamp® DNA mini kit (Qiagen, Hilden, Germany). SNP genotyping was conducted by direct sequencing. Polymerase chain reactions (PCRs) were conducted using primers specific for the BMP3 SNPs that were selected for analysis (rs13138132: sense, 5'-GTGTGACTCCAGGAGCCTATCT-3'; antisense, 5'-TGA GCTAACATTTTCTC CACAC-3'; product size, 376 bp; and rs3733549: sense, 5'-AGTTGTCCAGTGTCTGGAGGAT-3'; antisense, 5'-TCCCTGTAAGCTTGATACCACA-3'; product size, 351 bp). The products were sequenced using an ABI PRISM 3730XL analyzer (PE Applied Biosystems, Foster City, CA, USA). The sequenced data were analyzed using SeqMan II software (DNASTAR, Madison, WI, USA).

Statistical analysis. SNPStats (<http://bioinfo.iconcologia.net/SNPstats>) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA) were used to analyze the genetic data and the Hardy-Weinberg equilibrium (HWE). Associations between the SNP genotypes and PTC, and associations between the SNP genotypes and PTC subgroups, were estimated by computing the odds ratios (ORs)

Table II. Comparison of genotype and allele frequencies of BMP3 polymorphisms between control and PTC patients.

SNP	Model/allele	Genotype	Control		PTC		OR (95% CI)	P-value
			n	%	n	%		
rs13138132 (-1919C/A)	Codominant	CC	228	70.4	68	64.8	Ref.	
		CA	91	28.1	35	33.3	1.31 (0.81-2.13)	0.29
		AA	5	1.5	2	1.9	1.38 (0.26-7.46)	0.73
	Dominant	CC	228	70.4	68	64.8	Ref.	
		CA/AA	96	29.6	37	35.2	1.32 (0.82-2.12)	0.26
	Recessive	CC/CA	319	98.5	103	98.1	Ref.	
		AA	5	1.5	2	1.9	1.27 (0.24-6.79)	0.78
	Allele	C	547	84.4	171	81.4	Ref.	
		A	101	15.6	39	18.6	1.24 (0.82-1.86)	0.31
rs3733549 (Arg192Gln)	Codominant	GG	217	67.0	64	62.1	Ref.	
		GA	101	31.2	32	31.1	1.08 (0.66-1.78)	0.77
		AA	6	1.8	7	6.8	3.98 (1.24-12.73)	0.017 ^a
	Dominant	GG	217	67.0	64	62.1	Ref.	
		GA/AA	107	33.0	39	37.9	1.24 (0.78-1.99)	0.36
	Recessive	GG/GA	318	98.2	96	93.2	Ref.	
		AA	6	1.8	7	6.8	3.88 (1.23-12.25)	0.023 ^a
	Allele	G	535	82.6	160	77.7	Ref.	
		A	113	17.4	46	22.3	1.36 (0.93-2.00)	0.12

P-values were calculated from logistic regression analyses adjusting for age and gender. ^aStatistically significant values (P<0.05/2). BMP3, bone morphogenic protein 3; PTC, papillary thyroid cancer. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

and 95% CIs with logistic regression analyses, controlling for age and gender as covariables. In the logistic regression analysis for each SNP, models assuming codominant inheritance, dominant inheritance or recessive inheritance were used. The χ^2 test was used to compare allele frequencies between groups. To avoid chance findings due to multiple testing, the Bonferroni correction was applied by lowering the significance levels to P=0.025 (P=0.05/2) for the two SNPs.

Results

Genetic associations between BMP3 and PTC. The genotypic distributions of the SNPs examined in this study were in HWE (P>0.05; data not shown). Genetic associations between BMP3 and PTC were investigated. Multiple logistic regression analyses were conducted for the promoter SNP rs13138132 (codominant, CA vs. CC and AA vs. CC; dominant, CA/AA vs. CC; recessive, AA vs. CC/CA) and the missense SNP rs3733549 (codominant, GA vs. GG and AA vs. GG; dominant, GA/AA vs. GG; recessive, AA vs. GG/GA).

As shown in Table II, the genotype frequencies of rs3733549 were significantly different between the PTC patients and control subjects in the codominant (AA vs. GG) and recessive models (AA vs. GG/GA). In the codominant model, the frequencies of the GG and AA genotypes were 67.0 and 1.8% in the control group, and 62.1 and 6.8% in the PTC group, respectively. The AA genotype was associated with an increased risk of PTC (P=0.017, OR=3.98 and 95% CI=1.24-12.73). In the

recessive model, the frequencies of the genotypes containing the G allele (GG/GA) and the genotypes not containing the G allele (AA) were 98.2 and 1.8% in the control group and 93.2 and 6.8% in the PTC group, respectively. The frequency of the genotype not containing the G allele was significantly increased in the PTC patients compared with the control subjects (P=0.023, OR=3.88, 95% CI=1.23-12.25). rs13138132 was not significantly associated with PTC.

Genetic correlation between the SNPs and PTC patient subgroups. When we assessed the genetic correlation between the SNPs and PTC patient subgroups, the BMP3 SNP rs13138132 was significantly associated with PTC with extrathyroidal invasion in the codominant (CA vs. CC; P=0.006, OR=3.52 and 95% CI=1.44-8.62) and dominant models (CA/AA vs. CC; P=0.0023, OR=3.79 and 95% CI=1.56-9.21; Table III). The frequencies of genotypes containing the A allele (CA/AA genotypes) were increased in PTC patients with extrathyroidal invasion (49.1%) compared with PTC patients without extrathyroidal invasion (20.4%). Allele frequency analysis revealed that rs13138132 was associated with extrathyroidal invasion (P=0.004, OR=3.16, 95% CI=1.44-6.92), and that the frequency of the A allele was increased in PTC patients with extrathyroidal invasion (10.2 and 26.4% in PTC patients without and with extrathyroidal invasion, respectively). rs3733549 did not demonstrate any association with cancer size, tumor number, cancer location, extrathyroidal invasion, angiolymphatic invasion or cervical lymph node metastasis.

Table III. Comparison of genotype and allele frequencies of BMP3 polymorphisms bwtween PTC patients with and without extrathyroidal invasion.

SNP	Model/allele	Genotype	Extrathyroidal invasion				OR (95% CI)	P-value
			-		+			
			n	%	n	%		
rs13138132 (-1919C/A)	Codominant	CC	39	79.6	27	50.9	Ref.	0.006 ^a
		CA	10	20.4	24	45.3	3.52 (1.44-8.62)	
		AA	0	0	2	3.8	NA	
	Dominant	CC	39	79.6	27	50.9	Ref.	0.0023 ^a
		CA/AA	10	20.4	26	49.1	3.79 (1.56-9.21)	
	Recessive	CC/CA	49	100	51	96.2	Ref.	
		AA	0	0	2	3.8	NA	
	Allele	C	88	89.8	78	73.6	Ref.	0.004 ^a
		A	10	10.2	28	26.4	3.16 (1.44-6.92)	
	rs3733549 (Arg192Gln)	Codominant	GG	28	59.6	34	64.2	Ref.
GA			14	29.8	17	32	0.99 (0.41-2.39)	
AA			5	10.6	2	3.8	0.33 (0.06-1.89)	
Dominant		GG	28	59.6	34	64.2	Ref.	0.65
		GA/AA	19	40.4	19	35.8	0.82 (0.36-1.88)	
Recessive		GG/GA	42	89.4	51	96.2	Ref.	0.18
		AA	5	10.6	2	3.8	0.33 (0.06-1.84)	
Allele		G	70	74.5	85	80.2	Ref.	0.34
		A	24	25.5	21	19.8	0.72 (0.37-1.40)	

P-values were calculated from logistic regression analyses adjusting for gender and age. ^aStatistically significant values ($P < 0.05/2$). BMP3, bone morphogenic protein 3; PTC, papillary thyroid cancer. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; NA, not applicable.

Discussion

The results of the present study indicate an association between the BMP3 gene and PTC. The rs3733549 SNP of BMP3 was associated with PTC, and the AA genotype contributed to an increased risk of PTC. Additionally, the rs13138132 SNP was associated with extrathyroidal invasion in PTC patients. In particular, we identified that the frequency of the A allele was increased in PTC patients with extrathyroidal invasion.

Although the role of BMP3 in the pathogenesis of cancer is unknown, several studies have demonstrated that BMP3 participates in the development of various types of cancer (17-21). Loh *et al* (17) reported that in colon cancer cell lines with methylation-dependent BMP3 downregulation, cell colony growth suppression was observed following the reintroduction of BMP3 coding sequences. Methylation of the BMP3 promoter has also been described in subtypes of stomach (18), lung (20), pancreatic (19) and breast cancers (21) with downregulated BMP3 expression. Considering the results of these previous studies, we speculated that BMP3 is also involved in the development of PTC. In our study, we revealed a weak association between rs3733549 and PTC.

Additionally, the SNP rs13138132 in BMP3 was associated with extrathyroidal invasion in PTC patients, and the frequency

of the minor A allele was increased in PTC patients with extrathyroidal invasion. Extrathyroidal invasion is one of the most prominent prognostic risk factors for a poor clinical outcome in patients with PTC (22). As mentioned, BMP3 may negatively regulate cellular processes including migration, proliferation and differentiation (16-19). In addition, BMP3 gene expression has been identified to be downregulated with hypermethylation of the BMP3 promoter region in various types of cancer (17-19). The SNP rs13138132 is located in a predicted promoter enhancing region (<http://asia.ensembl.org/index.html>); thus, we speculated that rs13138132 affects promoter activity and methylation of the BMP3 gene, and that rs13138132 eventually contributes to extrathyroidal invasion in PTC. Additional studies are required to determine how BMP3 polymorphisms affect the pathogenesis of cancers, including PTC.

In conclusion, we obtained significant evidence to support an association between BMP3 and PTC. In particular, the AA genotype of rs3733549 was associated with the development of PTC, and the A alleles of rs13138132 were implicated as a risk factor for extrathyroidal invasion in PTC. Thus, our results suggest that BMP3 plays a role in the pathogenesis of PTC. Due to the relatively small number of subjects, our results should be validated by additional studies using larger sample sizes.

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