Missense Polymorphisms in XIAP-Associated Factor-1 (*XAF1*) and Risk of Papillary Thyroid Cancer: Correlation with Clinicopathological Features

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Abstract. *X-Linked inhibitor of apoptosis (XIAP)-associated factor-1 (XAF1) antagonizes XIAP-mediated caspase inhibition. XAF1 also serves as a tumor-suppressor gene, and loss of XAF1 expression correlates with tumor progression. This study investigated whether XAF1 missense single-nucleotide polymorphisms (SNPs) are associated with the development of papillary thyroid cancer (PTC) and their clinicopathological features in a Korean population. Eighty-nine cases of PTC and 276 controls were enrolled. Two missense SNPs [rs34195599 (Glu85Gly) and rs2271232 (Arg132His)] in XAF1 were genotyped using direct sequencing. The SNPStats, SNPAnalyzer, Helixtree, and Haploview version 4.2 programs were used to evaluate genetic data. Multiple logistic regression models were used to determine odds ratios (ORs), 95% confidence intervals (CIs), and p-values. Missense SNP rs34195599 was weakly-associated with the development of PTC (p=0.046 in genotypic distributions; p=0.048 in allelic distributions). For the clinicopathological features, rs34195599 was strongly related to multifocality [unifocality (A/G, 1.7%) vs. multifocality (A/G, 16.7%), OR=11.44, 95% CI=1.27-103.26, p=0.015 in genotypic distributions] [unifocality (G, 0.8%) vs. multifocality (G, 8.3%), OR=10.64, 95% CI=1.21-93.23, p=0.017 in allelic distributions] and location [one lobe (A/G, 1.6%) vs. both lobes (A/G, 19.2%), OR=15.63, 95% CI=1.62-150.46, p=0.008 in genotypic distributions] [one lobe (G, 0.8%) vs. both lobes (G, 9.6%), OR=13.30, 95% CI=1.51-116.82, p=0.009 in allelic distributions]. Our data suggest that the G allele of rs34195599 of XAF1 may be a risk factor for the clinicopathological features of PTC, especially for multifocality and location (both lobes).*

The incidence of thyroid cancer is low (approximately 0.5-10/100,000 persons), however, it is the most common endocrine malignancy. Although the prognosis of patients with thyroid cancer is generally good, thyroid cancer accounts for the majority of deaths due to endocrine cancer (1, 2). Papillary thyroid cancer (PTC) is the predominant type of all thyroid cancers and its incidence has increased during the past two decades in developing countries (3). Factors including radiation, diet, smoking, and hormones have been reported to be involved in the pathogenesis of thyroid cancer, and genetic predisposition has also been implicated as a risk factor for thyroid cancer development (4-6). Several researchers have investigated the relationship between PTC and single-nucleotide polymorphisms (SNPs) of candidate genes, including vascular endothelial growth factor-A (*VEGFA*) (7), protein tyrosine phosphatase, receptor type, J (*PTPRJ*) (8), WD repeat domain-3 (*WDR3*) (9), RAD52 homolog (Saccharomyces cerevisiae) (*RAD52*) (10), TNF receptor superfamily, member 6 (*FAS*)/FAS ligand (TNF superfamily, member-6) (*FASLG*) (11), interleukin-6 (IL6) (12), *IL10* (13), and IL11 receptor-alpha (*ILRA*) (14).

Apoptosis, programmed cell death, has a pivotal role in the development and progression of cancer. The inhibitor of apoptosis (IAP) family are cellular regulators of apoptosis. The IAP family conserves the baculoviral IAP (BIR) domains and is classified into the nucleotide-binding oligomerization domain containing-2 (*NLR*) family, apoptosis inhibitory protein (NAIP, also known as BIRC1),

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BIR repeat containing-2 (BIRC2, CIAP1), BIRC3 (CIAP2), X-linked inhibitor of apoptosis (XIAP, BIRC4), BIRC5 (SURVIVIN), BIRC6 (APOLLON), BIRC7 (LIVIN), and BIRC8 (ILP2). XIAP has three BIR domains and inhibits caspases (15, 16). XIAP-associated factor-1 (XAF1) interacts with XIAP and antagonizes the anti-caspase activity of XIAP. XAF1 also inhibits apoptosis by radiation, 5-fluorouracil, TNF, and TNF (ligand) superfamily, member-10 (TNFSF10, TRAIL) in cancer cells (17-19). Therefore, XAF1 is important in mediating apoptosis resistance of cancer cells. XAF1 expression is ubiquitous in normal tissues, but is low or undetectable in several cancer cell lines. XAF1 expression is reduced in human cancer tissues, and loss of XAF1 expression correlates with tumor staging and progression (20-22). Yang *et al*. reported that the overexpression of XAF1 induced apoptosis in about 80% tumor cells, but only in 5;10% normal cells (23). They suggest that XAF1 overexpression could selectively-induce apoptosis of cancer cells, and XAF may be effective for gene therapy of human cancer. The genetic role of XAF1 on the development and clinicopathological features of thyroid cancer is largely unknown.

In this study, we investigated whether two missense SNPs, rs34195599 (Glu85Gly) and rs2271232 (Arg132His), in the *XAF1* gene are associated with the development of PTC in a Korean population. We also assessed the relationships between two missense SNPs of *XAF1* and cliniopathological features, such as the size, number, location of tumors, extrathyroidal invasion, and lymph node metastasis.

Materials and Methods

Participants. Study participants consisted of patients with PTC (n=89, 23 males and 66 females) and controls (n=276, 113 males and 163 females). The mean ages of the PTC and control groups were 53.5±11.5 years (mean±SD) and 56.7±12.6, respectively (Table I). Patients with PTCs were recruited from among these visiting the Kyung Hee University Medical Center, Seoul, Republic of Korea. Controls were enrolled from healthy participants examined in a general health check-up program. Participants with any other types of cancer, thyroid disease, or any other severe disease were excluded. PTC diagnosis was confirmed by pathological examination. Patients with anaplastic carcinoma, follicular carcinoma, double-primary of papillary thyroid carcinoma and follicular carcinoma, follicular variant of papillary thyroid carcinoma, or nodular hyperplasia were excluded. Written informed consent was obtained from all participants. This study was approved by the Ethics Review Board of the Medical Research Institute, Kyung Hee University Medical Center, Seoul, Republic of Korea (Kmc IRB 1010-05).

To assess the relationship between *XAF1* SNPs and the clinicopathological characteristics of PTC, patients were divided into sub-groups according to the size (<1 cm and ≥1 cm), number (unifocality and multifocality), location (one lobe and both lobes) of tumors, extrathyroidal invasion (present and absent), lymph node metastasis (present and absent), and angiolymphatic invasion (present and absent). Demographic features of patients with PTC are summarized in Table I.

Table I. *Clinical features in patients with papillary thyroid cancer (PTC) and controls*.

	PTC (n=89)	Control (n=276)
Age (mean±SD, year)	53.1±11.5	56.7±12.6
Gender (male/female)	23/66	113/163
Size of tumor		
<1 cm	48	
≥1 cm	41	
Number of tumors		
Unifocality	59	
Multifocality	30	
Location of tumor		
One lobe	63	
Both lobe	26	
Extrathyroidal invasion		
Absent	44	
Present	45	
Cervical lymph node metastasis		
Absent	61	
Present	24	
Angiolymphatic invasion		
Absent	84	
Present	5	

SD: Standard deviation. Patients with PTC with inappropriate clinical data were excluded.

SNP selection and genotyping. For the selection among *XAF1* SNPs, we searched the missense SNPs of the *XAF1* gene in the SNP database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP, BUILD 132). SNPs with heterozygosity below 0.1 or minor-allele frequency (MAF) below 0.05 were excluded. Out of nine SNPs, there were seven SNPs with heterozygosity below 0.1 and MAF below 0.05.

Finally, two SNPs, rs34195599 (Glu85Gly) and rs2271232 (Arg132His), were selected. Peripheral blood samples from each participant were collected in EDTA-coated tubes and stored at –20˚C. DNA was extracted using a QIAamp® DNA mini kit (QIAGEN, Valencia, CA, USA) and SNP genotyping was determined by direct sequencing. Polymerase chain reactions (PCRs) were performed using the primers for two missense SNPs (Table II). PCR conditions for the reaction were: 94˚C for 30 sec; 58˚C for 30; 72˚C for 1 min; 72˚C for 7 min to terminate the reaction. The PCR products were sequenced by an ABI PRISM 3730XL analyzer (PE Applied Biosystems, Foster City, CA, USA). Sequencing data were analyzed using SeqManII software (DNASTAR, Madison, WI, USA).

Statistical analysis. The Hardy–Weinberg equilibrium (HWE) was estimated using SNPStats (http://bioinfo.iconcologia.net/index.php?module=Snpstats) for both the patient and control groups. Helixtree (Golden Helix Inc., Bozeman, MT, USA) and SNPAnalyzer (ISTECH Inc., Goyang, Korea) programs were used to analyze genetic data. Linkage disequilibrium (LD) block and haplotypes

Table II. *Primer sequences for each single-nucleotide polymorphism (SNP).*

SNP	Primer		Product size (bp)
	Forward (5'-3')	Reverse (5'-3')	
rs2271232 (Arg132His)	TAGTCTCCCTGGACCAGTCATT	GGCAACACAATGAGACTCTGTC	494
rs34195599 (Glu85Gly)	CCCAGTTTGTGTGCTTACCTTT	GGTGAAACCCCGTCTCTACTAA	455

bp: Base pair.

Table III. *Genotypic and allelic frequencies of X-linked inhibitor of apoptosis associated factor-1 (XAF1) singe-nucleotide polymorphisms (SNPs) in patients with papillary thyroid cancer (PTC) and controls.*

SNP	Genotype/allele	Controls		PTC		Model	OR (95% CI)	*p*-Value
		n	%	n	%			
rs2271232	G/G	238	86.2	75	84.3	Codominant-1	1.01 (0.48-2.11)	0.87
Arg132His	G/A	37	13.4	11	12.4	Codominant-2	7.10 (0.71-70.71)	0.05
	A/A	1	0.4	3	3.4	Dominant	1.22 (0.62-2.42)	0.57
						Recessive	7.09 (0.71-70.54)	0.07
						Log-additive	1.37 (0.76-2.47)	0.3
	G	513	92.9	161	90.4		1	
	A	39	7.1	17	9.6		1.39 (0.77-2.52)	0.28
rs34195599	A/A	270	90.8	83	93.3		1	
Glu85Gly	A/G	6	2.2	6	6.7		3.25 (1.02-10.36)	**0.046**
	G/G	0	0.0	0	0.0			
	A	546	98.9	172	96.6		1	
	G	6	1.1	6	3.4		3.17 (1.01-9.97)	**0.048**

OR: Odds ratio, CI: confidence interval. The *p*-values were calculated from logistic regression analyses adjusting for gender and age. Bold numbers indicate a significant association.

were calculated using Haploview version 4.2 (Daly Lab Inc., Cambridge, MA, USA). Multiple logistic regression models and the Fisher's exact test were performed to obtain odds ratios (ORs), 95% confidence intervals (CIs), and *p*-values. Data analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered significant.

Results

The genotypic and allelic frequencies of the studied two missense SNPs are presented in Table III. Multiple logistic regression analysis with adjustment for age and gender was performed; Codominant-1 (major allele homozygotes *vs*. heterozygotes), Codominant-2 (major allele homozygotes *vs*. minor allele homozygotes), Dominant (major allele homozygotes *vs*. heterozygotes+minor allele homozygotes), Recessive (major allele homozygotes+ heterozygotes *vs*. minor allele homozygotes), and Log-additive (major allele homozygotes *vs*. heterozygotes *vs*. minor allele homozygotes)(24). Missense SNP rs2271232 (CGC132CAC, Arg132His) of *XAF1* was not associated with PTC. Another missense SNP (rs34195599, GAG85GGG, Glu85Gly) was weakly related to the development of PTC in genotypic ($p=0.046$, OR=3.25, 95% CI=1.02-10.36) and allelic distributions ($p=0.048$, OR=3.17, 95% CI=1.01-9.97) (Table III). However, these significances disappeared upon Bonferroni correction.

Next, we assessed the relationship between *XAF1* SNPs and the clinicopathological features of PTC. Concerning the number of tumors (unifocality and multifocality), the rs34195599 SNP was associated with multifocality in genotypic ($p=0.0098$, OR=11.44, 95% CI=1.27-103.26; corrected *p* by the Fisher's exact test, $p^c=0.015$) and allelic frequency ($p=0.033$, OR=10.64, 95% CI=1.21-93.23; $p^c=0.017$). These significances remained after Bonferroni correction ($p<0.05$) (Table IV). The A/G frequency of rs34195599 was different between the unifocality and multifocality groups (1.7% *vs*. 16.7%). The G allelic frequency of rs34195599 was significantly higher in the multifocality group (8.3%) than in the unifocality group (0.8%) (Table IV).

Table IV. *Genotypic and allelic frequencies of X-linked inhibitor of apoptosis associated factor 1 (XAF1) singe nucleotide polymorphisms (SNPs) in patients with papillary thyroid cancer (PTC) with unifocality and PTC patients with multifocality.*

SNP	Genotype/ allele	Unifocality		Multifocality		Model	OR (95% CI)	p^c-Value (Fisher's exact *p*)	Bonferroni corrected *p*-Value
		n	%	n	%				
rs2271232	G/G	50	84.8	25	83.3	Codominant1	1.01 (0.48-2.11)	0.69	1.00
Arg132His	G/A	8	13.6	3	10.0	Codominant2	7.10 (0.71-70.71)	0.27	0.54
	A/A	1	1.7	2	6.7	Dominant	1.22 (0.62-2.42)	0.57	1.00
						Recessive	7.09 (0.71-70.54)	0.07	0.14
						Log-additive	1.37 (0.76-2.47)	0.3	0.60
	G	108	91.5	53	88.3		1		
	A	10	8.5	7	11.7		1.43 (0.51-3.96)	0.5	1.00
rs34195599	A/A	58	98.3	25	83.3		1		
Glu85Gly	A/G	1	1.7	5	16.7		11.44 (1.27-103.26)	**0.0098 (0.015)**	**0.03**
	G/G	0	0.0	0	0.0				
	A	117	99.2	55	91.7		1		
	G	1	0.8	5	8.3		10.64 (1.21-93.23)	**0.033 (0.017)**	**0.034**

OR: Odds ratio, CI: Confidence interval. The *p*-values were calculated from logistic regression analyses adjusting for gender and age. Bold numbers indicate a significant association.

Table V. *Genotypic and allelic frequencies of X-linked inhibitor of apoptosis associated factor 1 (XAF1) singe nucleotide polymorphisms (SNPs) in patients with papillary thyroid cancer (PTC) with one lobe and PTC patients with both lobes.*

SNP	Genotype/ allele	One lobe		Both lobes		Model	OR (95% CI)	p^c-Value (Fisher's exact *p*)	Bonferroni corrected *p*-Value
		n	%	n	%				
rs2271232	G/G	55	87.3	20	76.9	Codominant1	0.94 (0.22-4.04)	0.97	1.00
Arg132His	G/A	8	12.7	3	11.5	Codominant2	NA (0.00-NA)		
	A/A	0	0.0	3	11.5	Dominant	1.98 (0.59-6.64)	0.28	0.56
						Recessive	NA (0.00-NA)		
						Log-additive	2.42 (0.93-6.32)	0.06	0.12
	G	118	93.7	43	82.7		1		
	A	8	6.3	9	17.3		3.09 (1.12-8.51)	**0.029**	0.058
rs34195599	A/A	62	98.4	21	80.8		1		
Glu85Gly	A/G	1	1.6	5	19.2		15.63 (1.62-150.46)	**0.0046 (0.008)**	**0.016**
	G/G	0	0.0	0	0.0				
	A	125	99.2	2	90.4		1		
	G	1	0.8	5	9.6		13.30 (1.51 -116.82)	**0.02 (0.009)**	**0.018**

OR: Odds ratio, CI: confidence interval, NA: not applicable. The *p*-values were calculated from logistic regression analyses adjusting for gender and age. Bold numbers indicate a significant association.

Concerning the location of tumors (one lobe or both lobes), the rs2271232 SNP was weakly associated with the tumor in both lobes in allelic frequency (p=0.029, OR=3.09, 95% CI=1.12-8.51), but not in genotypic frequency. The A allelic frequency of rs2271232 was different between the group with tumor in one lobe and that with tumor in the both lobes (6.3% *vs*. 17.3%) (Table V). The rs34195599 SNP was strongly associated with the location in genotypic (p=0.0046, OR=15.63, 95% CI=1.62-150.46; p^c=0.008) and allelic frequencies (p=0.020, OR=13.30, 95% CI=1.51-116.82; p^c=0.009). These significances remained after Bonferroni correction (p<0.05) (Table V). The A/G frequency of rs34195599 was different between the group with tumor in one lobe and that with tumor in the both lobes (1.6% *vs*. 19.2%). The G allele frequency of rs34195599 was higher latter (9.6%) than in the one lobe group (0.8%) (Table V).

However, the rs2271232 SNP was weakly related to the size of PTC in the dominant model (p=0.048), while the rs2271232 and rs34195599 SNPs were not associated with extra-thyroidal invasion and lymph node metastasis of PTC (data not shown). No of the SNPs in both the PTC group and the control group had any deviation in HWE (p>0.05, data not shown). LD block between rs2271232 and rs34195599 was determined using Haploview version 4.2. Since the LD block was not made (D'=0.136 and r^2=0.003), the analysis of haplotypes was not performed. We also did not analyze angiolymphatic invasion of PTC because the numbers of patients with angiolymphatic invasion were comparatively few (n=5). Our data revealed that *XAF1* was associated with the clinicopathological features of PTC, and that the G allele of rs34195599 may be a risk factor for multifocality and bilobar tumor in this Korean population.

Discussion

As far as we are aware, this is first study on the association between *XAF1* and PTC in a Korean population. We found that a missense SNP (rs34195599, Glu85Gly) in the *XAF1* gene was associated with the clinicopathological features of PTC, especially multifocality and tumor location.

XAF1 is thought to be a tumor-suppressor gene and antagonizes XIAP-mediated caspase inhibition. Loss of *XAF1* has been observed in cancer cell lines and human cancer tissues (17-19, 23). Epigenetic alterations such as DNA methylation are related to the expression of XAF1 (25, 26). Several studies have demonstrated that loss of *XAF1* is associated with the development and progression of various types of cancer. Tu *et al*. reported that the restoration of XAF1 expression inhibited tumor growth, enhanced TRAIL-induced apoptosis, and prolonged the survival in mice (18). Kempkensteffen *et al*. found that low XAF1 expression was associated with tumor staging, tumor grading, and overall survival time in clear-cell renal cell cancer (20). In the study of Shibata *et al*. which investigated the patients with gastric adenocarcinoma, XIAP and XAF1 were not significantly associated with disease-specific survival (21). However, they showed that patients with a high expression of XIAP and low expression of XAF1 had a significantly poorer survival in a Japanese population. Huang *et al*. reported that the down-regulation of *XAF1* was significantly correlated with tumor staging and shorter survival times in Chinese patients with pancreatic cancer (22). The study by Augello *et al*. (27) revealed that an imbalance in *XIAP/XAF1* mRNA expression levels was related to the overall survival of patients with hepatocellular carcinoma in Italy. Therefore, increasing of XAF1 expression could be an important strategy for the gene therapy of cancer.

To our knowledge, there has been no reported genetic study of *XAF1*, and only a few studies of *XIAP* SNPs have been published. Ferretti *et al*. reported that 423Q in XIAP was a predisposing factor for the development of idiopathic periodic fever through its influence on monocyte function (28). Salzer *et al*. did not find any defects in the coding sequence of *XIAP* in 30 German patients with hypogammaglobulinemia (29). Kang *et al*. showed that 12 SNPs of *XIAP* did not significantly affect susceptibility to lung cancer in a Korean population (30). In our study, we found that the missense SNP rs34195599 was weakly-associated with the development of PTC. Moreover, the rs34195599 SNP significantly affected in the clinicopathological features of PTC, such as the number (unifocality *vs*. multifocality) and location (one lobe *vs*. both lobes). The G allelic (Gly85) frequency of rs34195599 was about 10-fold higher in patients with multifocal than in patients with the unifocality. The G allelic (Gly85) frequency of rs34195599 in the bilobar tumor group was increased 12-fold compared with unilobar group (9.6%). Therefore, we propose that rs34195599 could be a useful marker for the number and location of PTC and the G allele of rs34195599 may be a risk factor of PTC progression. Although PTC has a good prognosis, the multifocality and location of PTC are related to the indications for complete thyroidectomy and postoperative radiation.

XAF1 protein (UniProt ID, Q6GPH4) consists of 301 amino acids and comprises one XAF1 chain from 1 to 301 amino acids containing one TNF receptor-associated factor (TRAF)-type zinc finger domain from 22 to 99 amino acids (www.uniprot.org/uniprot). To date, the exact biological roles of the two missense SNPs rs34195599 and rs2271232 in the *XAF1* gene are obscure. Since many reports have demonstrated that amino-acid changes in proteins are related to the pathogenesis of human diseases, including cancer, it is conceivable that the two missense SNPs of *XAF1* examined in this study may affect the development or clinical features of human diseases. Our case control study has some limitations. The sample size of patients is small, and controls did not undergo the thyroid ultrasonography. Considering the relatively low incidence of thyroid cancer (0.5–10/100,000 persons), more studies with larger numbers of patients are needed to verify our results.

In conclusion, our data suggest that the G allele of rs34195599 in *XAF1* may be a risk factor for the clinicopathological features or multifocality and bilobar location of PTC in the Korean population.

Conflicts of Interest

The Authors indicate that no potential conflicts of interest exist.

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