Association of *IRAK1* Gene Polymorphism rs3027898 With Papillary Cancer Restricted to the Thyroid Gland: A Pilot Study

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Abstract. *Background/Aim:* The incidence of thyroid cancer has increased predominantly due to an increase in papillary thyroid cancer (PTC). Alteration of toll-like receptor function has been reported to play a crucial role in carcinogenesis. The aim of the present study was to investigate predisposition to PTC associated with genetic markers of toll-like receptor and interleukin-1 receptor pathways involving nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) stimulation. Specifically, the study focused on the following genes: interleukin-1 receptor-associated kinase 1 (*IRAK1*, rs3027898), NF- κ B inhibitor alpha (*NFKBIA*, rs696), NF- κ B subunit 1 (*NFKB1*, rs28362491), and microRNA-146a (*miR-146a*, rs2910164). *Patients and Methods:* Forty-eight unrelated patients with papillary cancer restricted to the thyroid gland and 93 healthy volunteers were enrolled in the study. *Results:* A strong statistically significant difference was observed between patients with PTC and controls for *IRAK1* rs3027898 variant. When the statistical analysis was replicated taking into account patient's sex, the rs3027898 A allele was revealed to be the risky variant in males. *Conclusion:* Additional studies in larger groups of patients of various origins are needed to validate these preliminary findings.

Thyroid cancer is the most common neoplasia of the endocrine system and accounts for 1% of all newly diagnosed cancer cases. Papillary thyroid carcinoma (PTC) is the most common malignant thyroid neoplasm, comprising up to 80% of all thyroid carcinomas (1). Notable progress has been made in the application of molecular markers to cancer diagnosis in thyroid nodules fine-needle aspirates (2). The most common genetic alteration found in 40-45% of PTCs is the B-Raf proto-oncogene mutation resulting in a valine-to-glutamate change at the residue 600 (V600E) of the *BRAF* protein, which is a serine/threonine kinase (3). However, even though the *BRAF* V600E mutation is a sensitive marker, it is not a specific index of PTC susceptibility and lethality (2). Recently, the advancements in genotyping methods have permitted more efficient patient management based on the combination of cytological and molecular testing techniques (2). Nevertheless, additional knowledge of the genetic basis of PTC is expected to improve the individualized management of patients with thyroid nodules and cancer.

Even though an increasing number of publications have shown that nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) plays a role in thyroid cancer, little is known on the role of genetic variants in genes of NF- κ B pathway in PTC susceptibility (4). NF- κ B is a transcription factor of genes related to apoptosis, inflammation and immune response. Interleukin-1 (IL1) receptor-activated kinase1 (*IRAK1*) plays a significant role in toll-like receptor and IL1 receptor pathways of NF- κ B activation (5, 6). Specifically, NF- κ B is inactivated by cytoplasmic trapping through the inhibitors of kappa B (I κ B) proteins (*e.g.* NF- κ B inhibitor alpha, *NFKBIA*). Kinases phosphorylate serine residues of the I κ B proteins and lead them for destruction *via* the ubiquitination pathway. Subsequently, NF- κ B factor is activated and regulates the expression of a variety of genes implicated in various biological events.

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Recently, the -94 insertion/deletion ATTG polymorphism in the NFKB1 promoter (rs28362491) was associated with an increased risk of PTC in Chinese patients (7). In addition, toll-like receptor-associated polymorphisms were associated with PTC in Koreans (8, 9). Furthermore, it was reported that a common variant in *miR-146a* reduces mature miRNA expression and predisposes to PTC (10). It is worth mentioning that *miR-146a* is a microRNA that targets *IRAK1* gene, of which the variant rs3027898 has been associated with immune diseases by our and other groups (10-14). Additionally, an emerging body of literature shows that altered *IRAK1* expression predisposes to inflammatory diseases, autoimmune diseases, solid tumors and hematological malignancies, which may explain the focus on IRAK1 inhibitors in preclinical and clinical studies (15, 16).

In the present study, we aimed to genotype DNA from patients suffering from PTC confined to the thyroid gland. Specifically, we explored the role of polymorphisms of four genes belonging to the toll-like receptor/IL1 receptor pathways, namely *IRAK1* [rs3027898, in the 3'-untranslated region (3'-UTR)], *NFKB1* (rs28362491), *NFKBIA* (rs696, in the 3' UTR), and *miR-146a* (rs2910164, in the sequence of miRNA precursor).

Patients and Methods

Forty-eight unrelated patients with PTC (13 males; mean age=51±12 years, range=20-74 years) were enrolled in the study. All patients had a PTC of less than 4 cm [up to T2 according to the American Cancer Society (17)] that was confined into the thyroid gland (no microscopic or macroscopic extra-thyroidal extension) and had no apparent lymph node spread (clinical or imaging-negative lymph nodes). Additionally, 93 healthy volunteers (59 males; mean age=50±18 years, range=17-85 years) with no personal or family history of neoplasia, chronic autoimmune or infectious diseases, were studied. This study adhered to the tenets of the declaration of Helsinki (version 2002) and approved was by the Ethics Committees of AHEPA University Hospital and Aristotle University of Thessaloniki (approval numbers 237/7-12-2015 - 351/1-3-2017). Written consent was obtained from each participant in the study.

Genomic DNA was extracted from peripheral blood lymphocytes using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Polymorphisms rs3027898 *IRAK1*, rs28362491 *NFKB1*, rs696 *NFKBIA*, and rs2910164 *miR-146a* were studied with restriction fragment length polymorphism assay (RFLP) as described previously (13, 18). All samples were run twice and random samples were analyzed by sequencing to qualify the genotyping data by the RFLP analysis.

SPSS statistical package (SPSS Inc., Chicago, IL, USA) was used to test differences in distribution of each polymorphism between patients with PTC and controls (Pearson's chi-square, Yates' chi-square if any expected frequency was below 1 or if the expected frequency was less than 5 in more than 20% of cells). Furthermore, the odds ratio (OR) with a confidence interval (CI) of 95% was calculated (reference allele *versus* the variant allele). A difference at $p \leq 0.05$ was considered as statistically significant.

Results

Polymorphisms rs696 and rs2910164 were in Hardy-Weinberg equilibrium in the control group ($p=0.0967$ and $p=0.8020$; respectively), while the rs28362491 variant was not ($p=0.0001$). The latter is in accordance with other European control groups which were not or were only approximately in equilibrium for the rs28362491 variant (19). Finally, the X-chromosome linked polymorphism rs3027898 was in Hardy-Weinberg equilibrium in the female control group, where the three genotypes existed ($p=0.6615$). More specifically, in the control group, 15 females carried the AA genotype, 16 the AC genotype, and 3 the CC genotype, while 39 males carried the A allele and 20 the C allele. Concerning the patients, 13 females carried the AA genotype, 19 the AC genotype, and 3 the CC genotype, while 13 male patients carried the A allele and none the C allele (Table I).

The distribution of the other studied polymorphisms rs28362491 (*NFKB1*), rs696 (*NFKBIA*), and rs2910164 (*miR-146a*) did not differ significantly between patients with PTC and controls (Table I). Strong statistically significant difference was observed between patients with PTC and controls in genotype's distribution of polymorphism rs3027898 located in the 3' UTR of *IRAK1* gene ($p=0.002$, Table I).

Taking into consideration the hemizygous state of rs3027898 polymorphism (X-linked) in males and the X-inactivation process in females (one active X-chromosome in female cells), we replicated the analysis of rs3027898 genotype's distribution in male patients *versus* male controls, and in female patients *versus* female controls. Regarding the female groups, the analysis of rs3027898 was not expanded to alleles, but restricted to genotypes because non-random X inactivation patterns were also reported for various other X-linked genes (20) which might cause biased grouping of heterozygous alleles in females. In these analyses, strong statistical difference was observed in the allelic distribution between male PTC patients *versus* male controls with the A allele being the risky variant ($p=0.033$, Table I). However, the confidence interval includes the value 1, which may seem to cancel the observed association. Nevertheless, we also mention the small sample size of our groups which may have affected the confidence interval width and be misleading (21).

Discussion

Thyroid cancer is a common malignancy of the endocrine system that requires early pre-operative diagnosis, especially in cases when the tumor is small and confined within the gland. In this context, we tried to identify in the blood, possible gene polymorphisms that might be used in the future as biomarkers in the earlier diagnosis and management

Table I. Statistical analysis of the three studied polymorphisms in patients with papillary thyroid cancer and controls.

Gene	SNP				Patients (N=48; male=13)	Controls (N=93; male=59)	p-Value	OR (95% CI)	p-Value	
NFKB1	rs28362491	Genotype	Ins		9 (19%)	11 (12%)	0.359	Referent 1.316 (0.803-2.157)	0.276	
			Ins/Del		34 (71%)	66 (71%)				
			Del		5 (10%)	16 (17%)				
		Allele	Ins		52 (54%)	88 (47%)				
			Del		44 (46%)	98 (53%)				
NFKB1A	rs696	Genotype	GG		15 (31%)	24 (26%)	0.79	Referent 1.153 (0.701-1.897)	0.575	
			GA		26 (54%)	54 (58%)				
			AA		7 (15%)	15 (16%)				
		Allele	G		56 (58%)	102 (55%)				
			A		40 (42%)	84 (45%)				
miR-146a	rs2910164	Genotype	GG		26 (54%)	53 (57%)	0.942	Referent 0.906 (0.515-1.596)	0.734	
			GC		19 (40%)	35 (38%)				
			CC		3 (6%)	5 (5%)				
		Allele	G		71 (74%)	141 (76%)				
			C		25 (26%)	45 (24%)				
IRAK1	rs3027898	Genotype	CC		3 (6%)	23 (25%)	0.002	Referent 0.071 (0.004-1.262)	0.033#	
			AC		19 (40%)	16 (17%)				
			AA		26 (54%)	54 (58%)				
		Allele	Male	C		0 (0%)	20 (34%)			
				A		13 (100%)	39 (66%)			
		Genotype	Female	CC		3 (9%)	3 (9%)			0.872#
				AC		19 (54%)	16 (47%)			
				AA		13 (37%)	15 (44%)			

NFKB1: Nuclear factor kappa-light-chain-enhancer of activated B-cells subunit 1; *NFKB1A*: nuclear factor kappa-light-chain-enhancer of activated B-cells inhibitor alpha; *IRAK1*: interleukin-1 receptor-associated kinase 1; OR: odds ratio, 95% CI: 95% Confidence interval. [#]Yates' correction.

of PTC. Our research focused on the toll-like receptor pathway, increasingly implied in the pathogenesis of solid tumors (22).

IRAK1 is a serine/threonine kinase that mediates toll-like receptor and IL1 signaling pathways, which activate *NF-κB*, increasing the expression of inflammatory cytokines. On the other hand, *miR-146a* which subsequently inhibits the expression of *IRAK1* protein, causes a negative feedback loop (23).

In the present study, a significant difference in the distribution of *IRAK1* polymorphism rs3027898 was observed between PTC patients and controls. Previously, the rs3027898 A allele was reported to be the risky variant in autoimmune thyroid diseases and in predisposition to Graves' disease (24). Taking into account that this variant is located on the X-chromosome, the statistical analysis was repeated according to sex due to the hemizygous state of the X-chromosome in males and the X-inactivation process in females. The strong statistical association with the A allele found to be the risky variant was confirmed in male patients, but not between female patients and controls. This may be accounted for by the difference in the expression of the protective rs3027898 C allele between male *versus* female controls: The rs3027898 C variant was found in the 20

hemizygous C male controls out of the 59 male controls and in 3 homozygous CC female controls out of 34 female controls. In heterozygous AC female controls (n=19), due to X inactivation process, which allele is eventually expressed is unknown. The potential high expression of the protective rs3027898 C allele in male controls due to the hemizygous state may also explain why PTC is three-times higher in women than in men (25).

Additionally, in previous studies, the expression of *IRAK1* mRNA was reported significantly lower in PTC clinical tissue samples than normal adjacent thyroid specimens, while that of *miR-146a* was reportedly increased (10, 26-28). It is worth mentioning that in a genetic association study, the rs2910164 GC heterozygous state was associated with PTC showing higher *miR-146a* expression than the homozygous rs2910164 GG state, while the homozygosity for rs2910164 CC was extremely rare among patients with PTC (10). However, the rs2910164 association with PTC was not replicated by our study, nor by other studies of Caucasian and Asian origin, nor in a recent meta-analysis (29-31).

In contrast, we revealed the association of *IRAK1* rs3027898 A allele with PTC in males. This finding might be compatible with the low *IRAK1* gene expression in PTC clinical tissue sample if we take into account that the rs3027898 A allele is

much more frequent compared to the rs3027898 C allele (0.8 A vs. 0.2 C in Caucasians derived from NCBI database according to HapMap-CEU project). Therefore, in the majority of *IRAK1* mRNA 3'-UTR sequences, where *miR-146a* acts and restricts *IRAK1* mRNA expression, the rs3027898 A allele is expected to exist. However, even though further studies are needed to confirm this hypothesis, the targeting of *miR-146a/IRAK1* axis as a potential approach for PTC management has already been suggested (32).

In conclusion, to our knowledge, this is the first study to report an association of the *IRAK1* gene polymorphism rs3027898 with PTC. The present study, which was designed to include only small tumors (less than 4 cm) with no extra-thyroidal extension nor lymph node spread, indicates that *IRAK1* rs3027898 might have potential use as a biomarker for the early identification of PTC risk before its local spread. However, this was only a pilot study (33) restricted to small patient and control groups of Greek origin, and therefore, we highlight the need for additional studies in larger groups of patients of various origins to validate this finding. Additionally, functional analyses, which evaluate *IRAK1* mRNA/protein levels in patients with PTC and controls are needed so as to clarify the potential use of *IRAK1* in early diagnosis or management of this disease.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in regard to this study.

Authors' Contributions

A. Chatzikiyriakidou conceived and designed the study, performed the research, the data analysis and the literature review, and wrote the manuscript. A. Chorti performed sample collection. Th. Papavramidis pointed out cases and controls, and edited and reviewed the article.

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