

A PILOT STUDY OF *ANXA2*, *MED12*, *CALM1* AND *MAPK1* GENE VARIANTS IN PRIMARY HYPERPARATHYROIDISM

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ABSTRACT

Primary hyperparathyroidism (PHPT) is a common endocrine disorder characterized by the overactivity of the parathyroid glands. While a few genes have been linked to a predisposition for PHPT, the genetic foundation of the disease remains unclear, despite it being the third most prevalent endocrine disorder. This pilot study aimed to investigate, for the first time, the potential association between specific variants in Annexin A2 (*ANXA2*-rs7170178, rs17191344, rs11633032), Mediator Complex Subunit 12 (*MED12*-rs1057519912), Calmodulin 1 (*CALM1*-rs12885713), and Mitogen-Activated Protein Kinase 1 (*MAPK1*-rs1057519911) genes with PHPT. Previous expression analyses have indicated that the proteins related to these genes are involved in parathyroid adenomas or PTH signaling. Fifty unrelated PHPT patients and an equal number of healthy controls were enrolled in the study. Genotyping was conducted using the polymerase chain reaction - restriction fragment length polymorphism assay. Statistical analysis was performed to assess the con-

nection between genetic variants and PHPT. Our results revealed no significant differences in genotypes' or alleles' distributions of any of the studied variants between PHPT patients and controls. These findings suggest that these variants may not be linked to PHPT in the studied population. This pilot study, focusing on a Caucasian group of PHPT patients, contributes to the existing genetic data for future meta-analyses, which will provide a more precise definition of the genetic factors associated with PHPT susceptibility worldwide.

Keywords: *ANXA2*, *CALM1*, *MAPK1*, *MED12*, genetic variants, primary hyperparathyroidism

INTRODUCTION

Parathyroid hormone (PTH) acts as an important regulator of calcium homeostasis in the human body [1]. The importance of PTH is reflected by the wide range of functions that calcium performs, as it participates in cell signaling, neural and muscular function, hormone release and regulation, and bone metabolism [2]. PTH increases the reabsorption of calcium in the kidney and the gastrointestinal tract while at the same time enhances the release of calcium from the bone reservoir by indirectly stimulating osteoclasts. Finally, PTH stimulates the conversion of 25-hydroxy vitamin D into 1,25-dihydroxy vitamin D (calcitriol), which is the active form of vitamin D and is released into the circulation [1].

Primary hyperparathyroidism (PHPT) is a prevalent endocrine disorder distinguished by the independent secretion of PTH as a result of overactivation of the parathyroid

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glands [3]. It is the third most common endocrine disorder after diabetes and thyroid disease, with prevalence between 0.1-0.4%. The number of diagnoses increases with age, peaking at 50-60 years, and it is more likely to occur in females. For the time being, PHPT is diagnosed by abnormalities in PTH and blood calcium levels [4].

Almost 90% of the patients with PHPT are found to have sporadic, non-familial, and non-syndromic disease. Sporadic PHPT is usually caused by a single gland adenoma (85%) but may also be caused by hyperplasia of all four glands (10%). Double adenomas (2-5%) and parathyroid carcinomas (<1%) account for the least common causes of the disease. Several genes and pathways have been implicated in PHPT. These include genes involved in cell cycle regulation, Wnt/ β -catenin signaling pathway, cellular growth, proliferation, tissue repairing, homeostasis, and apoptosis [5]. However, the genetic basis of PHPT is still under investigation.

In the present study, four genes previously reported to be involved in parathyroid adenomas or PTH signaling were analyzed for their association with PHPT. These genes are Annexin A2 (*ANXA2*), Mediator Complex Subunit 12 (*MED12*), Calmodulin 1 (*CALM1*), and Mitogen-Activated Protein Kinase 1 (*MAPK1*).

The *ANXA2* gene (15q22.2) encodes the ANXA2 protein, a calcium-regulated phospholipid-binding protein that has been found upregulated in some tumor cells, affecting cell survival and mediating interactions between intercellular and extracellular microenvironments. It performs crucial roles in tumor progression, especially in the invasion and metastasis of tumor cells [6]. In addition, increased expression of ANXA2 has been reported in parathyroid adenomas [7, 8].

The *MED12* gene (Xq13.1) is involved in gene regulation, as it serves as an essential component of the transcription mechanism of RNA polymerase II [9]. In general, *MED12* variants are common in neoplasms and benign tumors, while upregulation of *MED12* has been observed in parathyroid adenomas [10,11].

CALM1 (14q32.11) encodes one of the three calmodulin proteins, which are small calcium-sensitive proteins that rapidly transmit information about changes in calcium concentration, regulating gene expression in neurons and potentially shaping cardiac action in heart cells [12]. In parathyroid adenomas, calmodulin has been reported to inhibit PTH secretion [13].

Finally, the *MAPK1* gene (22q11.22) encodes a member of the MAP protein kinase family. It is also known as extracellular signal-regulated kinase 2 (ERK2) and has been strongly associated with proliferation, differentiation, and signaling regulation in osteoblasts [14]. The MAPK1/ERK2 protein is a key component of the Ras-Raf-MEK-

ERK and c-Jun N-terminal kinases (JNK) signaling pathways, which are downstream targets of PTH [14,15].

All these genes may have a role in PHPT predisposition due to their involvement in tumorigenesis in parathyroid glands and PTH signaling pathways. No studies were reported to test the association of genetic variants of *ANXA2*, *MED12*, *CALM1*, and *MAPK1* genes with PHPT predisposition, which is the reason why this pilot study was conducted. Genetic variants in *ANXA2* (rs7170178 A>G, rs17191344 A>G, and rs11633032 G>A; all downstream of the gene), *MED12* (rs1057519912; exonic: C>G, T), *CALM1* (rs12885713; intronic: C>T), and *MAPK1* (rs1057519911, exonic: C>T), previously described as variants with clinical relevance in several diseases, were studied as predisposing factors to PHPT pathogenesis.

MATERIALS AND METHODS

Fifty unrelated patients with primary hyperparathyroidism (PHPT) (2 males and 48 females, 56.1 ± 13.9 years) and an equal number of ethnically matched healthy volunteers (8 males and 42 females, 50.6 ± 18.4 years) were recruited for the study. The diagnosis of PHPT was confirmed by the elevated levels of parathyroid hormone (PTH) and calcium in blood serum, as well as through imaging methods such as sonography, 99mTc-sestamibi scintigraphy, and 4D-CT validated by histological examinations [16]. The control group had no personal or family history of chronic autoimmune or neoplastic diseases. Since this study on the association of the studied variants with PHPT was conducted for the first time, it was not possible to determine the standardized effect size to be used before the pilot trial. Therefore, the sample size was calculated with a 90% confidence level and a probability of 0.05 [17] following the suggested standards for pilot studies [18]. The study protocol was approved by the Ethics Committees of the Aristotle University of Thessaloniki, and written informed consent was obtained from each patient.

Genomic DNA was extracted from peripheral blood lymphocytes using the PureLink Genomic DNA Kit (Invitrogen) following the manufacturer's protocol. The samples were genotyped using the polymerase chain reaction - restriction fragment-length polymorphism (PCR-RFLP) assay. The primer pairs used for amplification of each region are shown in Table 1. Amplified fragments were then digested with appropriate restriction enzymes (New England Biolabs - Table 1), following the manufacturer's instructions, and visualized after electrophoresis on 3% agarose. All samples were run twice using RFLP analysis confirming the credibility of the results. This methodology is both time- and cost-saving for a pilot study like the pre-

Table 1. Primer sequences, restriction enzymes, and restriction digestion patterns were used for genotyping of the studied genetic variants.

Gene	Variant	Primer sequence (5'→3')	Amplicon size	Restriction Enzyme	Restriction digestion pattern
<i>ANXA2</i>	rs7170178	F: 5'-TTCACAGCAGTTCAAAATAC-3' R: 5'-CTGGGTTTCCAGAGATGGAA-3'	550bp	HpyCH4V	G: 338bp/106bp/72bp/34bp A: 195bp/143bp/106bp/72bp/34bp
	rs17191344	F: 5'-TGCAAACAGGCGCCACTAAA-3' R: 5'-CAGACATGAGGCCAAGGAAC-3'	280bp	Hpy99I	A: 94bp/186bp G: 139bp/94bp/47bp
	rs11633032	F: 5'-CAACAAGCATGGGGTTGC-3' R: 5'-GTTGACATTGCCCTTCGCTT-3'	131bp	BceAI	G: 123bp/ 8bp A: 131bp
<i>MED12</i>	rs1057519912	F: 5'-ACAAGCCTACAGTAGGAATCC-3' R: 5'-TGGCACCCTCCCTTCCTAC-3'	143bp	SmlI	C: 94bp/49bp G/T: 143bp
<i>CALM1</i>	rs12885713	F: 5'-GGGATACGGCGCACCATATAT-3' R: 5'-GGTACCTCCGATGCCGCTG-3'	179bp	HpyCH4V	T: 144bp/35bp C: 179bp
<i>MAPK1</i>	rs1057519911	F: 5'-TGGCTGATCTATGTCCCTGA-3' R: 5'-CACACAAGAGGATTGAAGTAG-3'	122bp	MnII	C: 63bp/59bp T: 122bp

Table 2. Statistical analysis of rs7170178 (*ANXA2* gene) and rs12885713 (*CALM1* gene) variants between PHPT patients and controls.

Gene: Genotypes of the studied variant	Patients	Controls	Statistical model	OR (95%CI)	p-Value	HWE in control group (p-value)
<i>ANXA2</i>: rs7170178						
AA	18	22	Additive (AA vs. AG vs. GG)		0.45	0.78
AG	29	23				
GG	3	5				
AA	18	22	Homozygous (GG vs. AA)	0.73 (0.15-3.49)	1	
GG	3	5				
AA	18	22	Heterozygous (AG vs. AA)	1.54 (0.67-3.53)	0.31	
AG	29	23				
AA	18	22	Dominant (AG+GG vs. AA)	1.4 (0.62-3.11)	0.41	
AG+GG	32	28				
AA+AG	47	45	Recessive (GG vs. AA+AG)	0.57 (0.13-2.55)	0.71	
GG	3	5				
A	65	67	Allelic (G vs. A)	1.09 (0.61-1.96)	0.76	
G	35	33				
<i>CALM1</i>: rs12885713						
CC	20	14	Additive (CC vs. CT vs. TT)		0.39	0.98
CT	19	25				
TT	11	11				
CC	20	14	Homozygous (TT vs. CC)	0.7 (0.24-2.06)	0.52	
TT	11	11				
CC	20	14	Heterozygous (CT vs. CC)	0.53 (0.21-1.32)	0.17	
CT	19	25				
CC	20	14	Dominant (CT+TT vs. CC)	0.58 (0.25-1.35)	0.21	
CT+TT	30	36				
CC+CT	39	39	Recessive (TT vs. CC+CT)	1 (0.38-2.57)	1	
TT	11	11				
C	59	53	Allelic (T vs. C)	0.78 (0.45-1.37)	0.39	
T	41	47				

sent one. However, other genotyping methods can be utilized in subsequent larger-scale studies if the results of the present study indicate the feasibility of such an approach. Pearson's chi-square test was used to examine possible deviations of genotype distributions from the Hardy-Weinberg equilibrium (HWE) in the control group. Differences in variant distribution between PHPT patients and controls were tested under six models of genetic association: homozygote, heterozygote, dominant, recessive, allelic, and additive using Pearson's chi-square test. Fisher's exact test was used when expected values were less than 5. Additionally, the odds ratio (OR) with a 95% confidence interval (CI) was calculated (reference allele vs variant allele). A difference at $p \leq 0.05$ was considered statistically significant in all statistical tests. All analyses were performed using the SPSS statistical package (SPSS Inc.).

RESULTS

The study group mainly included female PHPT patients, which can be excused by the female preponderance of the primary PHPT adenoma [19].

Three variants (*ANXA2*: rs17191344, rs11633032, *MAPK1*: rs1057519911) were found to be monomorphic for the wild-type alleles in both patients and controls. Additionally, the genotype distribution of the *MED12* rs1057519912 variant did not differ between PHPT patients and controls, with 2 patients and 2 control subjects being heterozygous. As a result, these four variants were not included in further statistical analyses.

The genotypic distribution of *ANXA2* rs7170178 and *CALM1* rs12885713 variants in PHPT patients and controls is displayed in Table 2. The distribution of genotypes was in line with Hardy-Weinberg equilibrium in the control group. No statistically significant different distributions of rs7170178 and rs12885713 genotypes or alleles were found between PHPT patients and controls (Table 2).

DISCUSSION

PHPT is a prevalent endocrine disorder characterized by the excessive functioning of the parathyroid glands [3]. The genetic basis of PHPT is only partially understood, and genetic variants have emerged as potential contributors to the development and progression of the disease affecting the genes' expression. This comprehensive study aimed to investigate the association between specific variants of *ANXA2*, *MED12*, *CALM1*, and *MAPK1* genes with PHPT.

The genes investigated in this study have been implicated in tumorigenesis and have shown associations with various types of cancer and parathyroid malignancies. *ANXA2* is involved in tumor progression, invasion,

and metastasis and its upregulation has been reported in parathyroid adenomas and several types of cancer [7, 8]. *MED12* variants were frequently observed in various cancer types, while overexpression has been related to parathyroid adenomas [11, 20]. *CALM1* was reported to participate in calcium signaling [12], making it a potential candidate for association with PHPT. Moreover, *CALM1* has been related to an inhibitory effect on PTH secretion in parathyroid adenoma [13]. Finally, *MAPK1/ERK2* has been described as a key component of signaling pathways regulated by PTH secretion and has been related to cell proliferation, differentiation, and survival [15].

In previous studies, the minor alleles of *ANXA2* rs7170178, rs17191344, and rs11633032 gene variants have been reported to reduce *ANXA2* gene expression and to down regulate *ANXA2* signaling [21, 22]. Specifically, the minor alleles create repressor-binding protein sites for transcription factors that contribute to reduced *ANXA2* gene expression [21, 22]. The variants rs17191344 and rs11633032 have been associated with coronary disease risk in Caucasians through increasing low-density lipoprotein cholesterol levels, while rs7170178 has been associated with osteonecrosis in sickle cell disease in Latin and Indian patients [21–23]. Moreover, *MED12* rs1057519912 and *MAPK1* rs1057519911 have been identified as hotspots in cancer [24]. In addition, rs12885713 in the promoter region of the *CALM1* gene has been reported to affect the transcription of the gene [25]. This variant has been studied for its association with osteoarthritis, but the results were contradictory [26, 27]. The meta-analysis including studies stratification by ethnicity in the analyses revealed that the rs12885713 variant increases the risk of osteoarthritis among Asians [26]. Furthermore, rs12885713 has also been associated with double curve and lumbar curve adolescent idiopathic scoliosis in Chinese patients [28, 29].

In a previous study, we have reported that *ANXA2*, *MED12*, and *MAPK1* proteins have positive staining in the immunohistochemical study of sporadic parathyroid adenomas in varying intensity and allocation percentages [30]. Due to technical issues in protocol establishment the protein *CALM1* has not been included in that study. In the present pilot genetic association study, a total of 50 unrelated PHPT patients and an equal number of healthy controls were genotyped for *ANXA2* (rs7170178, rs17191344 and rs11633032), *MED12* (rs1057519912), *CALM1* (rs12885713) and *MAPK1* (rs1057519911) genetic variants. The variants rs17191344 and rs11633032 of the *ANXA2* gene and the rs1057519911 of the *MAPK1* gene were found to be monomorphic which is in accordance with the very low frequency of their minor alleles reported in the NCBI database for Caucasians. However, these variants were initially selected to be studied based on

their reported positive association with transcription levels of *ANXA2* gene and as a cancer variant hotspot of *MAPK1* gene [21, 22, 24]. Regarding the variants rs7170178 *ANXA2*, rs1057519912 *MED12*, and rs12885713 *CALM1* no significant association was observed in genotypes or alleles distributions between PHPT patients and controls.

Due to the reported female preponderance of PHPT adenoma [19], the study group mainly included female PHPT patients. The present study is a pilot one and sets the initial step in exploring a novel intervention. Pilot results can inform about the feasibility and identify modifications needed in the design of a larger study testing the same hypothesis [31, 32]. It is worth mentioning that it was reported that power analyses should not be presented in an application in case of a pilot study which does not propose inferential tests. Instead, a pilot sample size is based on the pragmatics of recruitment and the necessity for examining the feasibility [31, 32].

The lack of significant associations between the studied genetic variants and PHPT of our study may be attributed to several factors, including the genetic heterogeneity of PHPT [33]. PHPT is a complex disorder influenced by both genetic and environmental factors, and multiple genetic variants likely contribute to its development. It is estimated that 60% of the variation in PTH concentration is genetically determined [34], and therefore several genetic variants have thus far been associated with PHPT pathogenesis causing among others disturbances in calcium regulation or cell signaling [35–38]. The sample size of our study is small, but it follows the suggested standards for pilot studies, which try to find preliminary evidence and tendencies of the studied variants' associations [18]. Additionally, the lack of significant genetic associations in this study is restricted to the studied genetic variants and does not reject the possible association between other variants of *ANXA2*, *MED12*, *CALM1*, and *MAPK1* genes with PHPT predisposition.

However, the publication of negative findings is as important as publishing statistically significant findings to overcome the issue of publication bias, which results from the preferential publication of positive associations and the reduced likelihood of negative findings being reported [39–41]. Even though the reported associations between gene variants and disease could have tremendous importance for the prevention, prediction, and treatment of diseases, commonly there is an irreproducibility of the results, and the majority of these associations are not robust [42]. Preliminary studies based on random small sample groups have been proved of great importance as many times the results of genome-wide association studies have limited clinical predictive value and other limitations [43,44]. Consequently, the negative associations, as these of the present

study, offer to decide if it is advantageous to investigate the above-mentioned variants as risk factors in disease predisposition especially in complex disorders such as PHPT [34]. It is worth mentioning that given the small sample size, the study may be underpowered to detect subtle associations. However, there are many reasons for the significance of pilot studies' results in the scientific community such as assessing the feasibility of a survey, assessing whether the research protocol is realistic and workable, and developing a research question and research plan [45].

Undoubtedly, understanding the genetic basis of PHPT can provide valuable insights into disease mechanisms and potentially guide the development to personalized treatment strategies. Genetic association studies of both positive and negative results can be proven a valuable resource in the struggle to understand and treat diseases since the conclusions should not be drawn from a single report. Positive and negative associations of other similar studies add to the pool of genetic data for their future meta-analyses concluding with more accuracy.

Declaration of Interest: The authors report no conflicts of interest.

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