INITIAL SCREENING OF THE RS104893657 VARIANT OF THE *PAX8* GENE IN WOMEN WITH HYPOTHYROIDISM FROM NORTHEASTERN SLOVAKIA

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SUMMARY

Objective: Thyroid diseases are among the most common endocrinopathies and metabolic disorders. Hypothyroidism is caused by insufficient production of thyroid hormones with a higher prevalence in women. Causes for the development of endocrine diseases may be mutations in genes that encode peptide hormones. The aim of this scientific study was to determine the genotype and allele frequencies of the rs104893657 variant of the *PAX8* gene and to determine the genotype versus phenotype association.

Methods: The study population consisted of 135 women from northeastern Slovakia who were divided on the basis of screening into two groups: a control group without diagnosed hypothyroidism (CG = 67) and a group of women with hypothyroidism (HY = 68). Biochemical markers – thyroid-stimulating hormone (TSH), prealbumin (PREA), calcium (Ca), phosphorus (P), and alkaline phosphatase (ALP) were determined using Cobas Integra 400 plus, Cobas e411 analysers (Roche). Genotyping was performed using TaqMan® SNP Genotyping Assay instrument 7500 Fast Real-Time PCR Systems (Applied Biosystem).

Results: Student's t-test revealed a statistically significant difference between CG and HY in biochemical parameters: TSH ($p < 0.001$), P ($p = 0.008$). By Chi-square test we found no statistically significant difference in the representation of genotypes ($p = 0.788$) in the rs104893657 polymorphism of *PAX8* gene. The T allele was not associated with hypothyroidism in Slovak women ($p = 0.548$). In CC genotype we found statistically significant difference between CG and HY in parameters TSH ($p < 0.001$) and P ($p = 0.006$).

Conclusion: The mutant T allele was detected at low frequency in both groups of women studied. The association of the T allele with the development of hypothyroidism in Slovak women was not confirmed. The results of this work provide initial information on the distribution of genotypes and alleles in the studied variant of *PAX8* gene in the Slovak female population.

Key words: hypothyroidism, women, PAX8 gene, marker, phenotype

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INTRODUCTION

Diseases of the thyroid gland are among the most frequent disorders of the endocrine glands. They affect all populations worldwide. A key determinant of thyroid disease risk is iodine nutrition (1). Hypothyroidism is a common health problem that is caused by insufficient production of thyroid hormones. The disease is associated with various symptoms such as fatigue, bradycardia, depression, obesity, and hyperlipidaemia (2). The worldwide prevalence of the disease is estimated to be 1.5 cases per 1,000 individuals. The incidence of thyroid problems including hypothyroidism in the Slovak Republic is approximately 5–10%. A higher likelihood of thyroid disorders is in women, with an average of 10–15% (3, 4). The epidemiology of thyroid disorders is influenced by factors such as ageing, smoking, genetic predisposition, endocrine disruptors, ethnicity, etc. (1). Hypothyroidism on autoimmune basis is one of the frequent diagnoses within thyroid diseases. Autoimmune thyroidopathies are polygenic conditioned diseases (5). Molecular genetic diagnosis of diseases of the endocrine system is of great importance. Genetic causes of the development of endocrine diseases can be mutations in genes encoding peptide hormones, mutations in genes for nuclear or membrane receptors and for binding proteins (6). Candidate genes that affect thyroid physiological processes, genesis and phenotypic variations include *HEX, HNF3-β, FGFR, TTF-2, NKX2.1, NKX2.5, HOXA3, PAX8* (7). *PAX8* is a transcription factor involved in organogenesis during embryonic development. Its regulatory role is also known in kidney, CNS, eye, thyroid, and Müllerian duct

development (8, 9). Mutations in this gene are associated with thyroid dysgenesis, follicular carcinomas, and atypical follicular thyroid adenomas (10, 11). The aim of our scientific study was to determine the representation of genotypes and alleles of the rs104893657 *PAX8* gene polymorphism in a population of Slovak women with hypothyroidism and in a control group of women. To find out whether there are statistically significant differences in the individual genotypes of the rs104893657 *PAX8* gene polymorphism between the control group of women and the group of women diagnosed with hypothyroidism in terms of monitored biochemical markers – thyroid-stimulating hormone (TSH), phosphorus (P), calcium (Ca), alkaline phosphatase (ALP), and prealbumin (PREA), and anthropometric parameters (body weight and height, waist and hip circumference, BMI, and WHR index).

MATERIALS AND METHODS

The study population consisted of 135 women from the district of northeastern Slovakia. The women were divided on the basis of clinical screening into two groups: a control group without diagnosed hypothyroidism (CG = 67) and a group of women with hypothyroidism (HY = 68). We excluded subjects with hysterectomy, ovariectomy, diagnosed with diabetes mellitus, liver disease, or confirmed tumor. The group of women with hypothyroidism included newly diagnosed patients without medical therapies. The research was conducted in the period from April to December 2022. The scientific study was conducted on the basis of the approval of the Ethics Committee of the University of Prešov No. 2/2013. The study was conducted in accordance with the ethical principles according to the Declaration of Helsinki and after obtaining written informed consent from all individuals.

Anthropometric parameters were measured as follows: body height (cm) – wall scale with a centimetre scale, body weight (kg) – digital personal scale (Möve Frottana, Germany) with an accuracy of 100 g, waist circumference (cm) and hip circumference (cm) were measured with a textile tape measure, taking into account the average of two consecutive measurements. We calculated BMI (kg/m^2) using the formula body height (m^2)/body weight (kg), and waist-hip ratio (WHR) as the ratio of waist circumference (cm) to hip circumference (cm).

Biochemical markers – thyrotropic hormone, calcium, phosphorus, alkaline phosphatase, and prealbumin were determined in blood serum. A peripheral blood sample was collected after fasting in the morning in a 6 ml BD Vacutainer® tube. Peripheral blood was centrifuged (Hettich universal 320R centrifuge, Germany) at 6,000 rpm for 10 minutes. Using a Cobas Integra 400 plus biochemical analyser (Roche, Switzerland), we analysed the following in the blood serum: Ca, P, ALP, and PREA. We determined TSH levels using the Cobas e411 immunochemical analyser (Roche, Japan).

For molecular genetic analysis, a peripheral blood sample was collected into BD Vacutainer® tubes (3 ml) with anticoagulant. Genomic DNA was isolated using the commercial kit NucleoSpin® Blood (Macherey-Nagel, Germany). Genotyping was performed using TaqMan genotyping SNP assay (C__27541358_10) (Thermo Fisher Scientific, Waltham, MA, USA) via standard protocol. Florescence was detected by Real-Time PCR using a 7500 Fast Real-Time PCR Systems instrument (Applied Biosystem, CA). The mutant genotype was verified by repeat measurements, then used as a positive control.

Statistical analysis of the data was performed using Statistica ver. 12. Among the statistical tests, Student's t-test was used to determine the statistical significance of differences in individual parameters between the compared groups. The Kruskal-Wallis test was used for non-parametric analysis of variance to detect differences in mean values between multiple sets.

To statistically evaluate the results of genotype and allele frequency representation between the control group and the group of women with hypothyroidism, we used the chi-squared test. MedCalc statistical software (2022) and Social Science Statistical (2022) calculator were used to calculate the odds ratio (OR) and confidence interval (CI). Pearson correlation coefficient was used for correlation analysis. We set $p < 0.05$ as statistically significant.

RESULTS

In Table 1 we present the mean values of anthropometric and biochemical parameters in the studied groups of women. Based on statistical analysis using Student's t-test, we found no statistically significant difference between the control group and the group of women diagnosed with hypothyroidism in the anthropometric parameters studied. On the basis of BMI index assessment, we categorized our groups (CG, HY) as overweight. The mean value of WHR index was 0.85 (±0.11) in CG and 0.89 (±0.15) in HY, which we classified as a risk group for the development of CVD. The resulting mean values of thyroid associated biochemical parameters (TSH and prealbumin), mineral elements (Ca, P), and enzymatic activity (ALP) in both CG and HY groups of women were within the range of reference values. Statistical analysis using Student's t-test revealed statistically significant difference between CG and HY in biochemical parameters: TSH ($p < 0.001$), P ($p = 0.008$). Through correlation analysis, we found statistically significant relationship ($p < 0.05$) between TSH and body height ($r = 0.371$), TSH and body weight ($r = 0.285$), ALP and age ($r = 0.314$) (positive correlation); BMI and prealbumin ($r = -0.244$) (negative correlation) (Fig. 1). We also investigated the representation of genotypes and alleles of rs104893657 *PAX8* gene polymorphism in the control group and the group of women diagnosed with hypothyroidism. In Table 2 we present their relative and absolute representation. The CC genotype was present in the highest frequency in both groups of women studied. Chi-square test did not reveal a statistically significant difference in the representation of genotypes ($p = 0.788$). In the polymorphism studied, the mutant allele was the T allele (CG 5.22%, HY 4.41%). We did not detect a possible association of the T allele with hypothyroidism disease using the online MedCalc calculator ($p = 0.755$). In Table 3, we present the mean values of anthropometric and biochemical parameters in each genotype in the two groups of women studied. We do not report values for the TT genotype because this genotype was detected in only two women in CG and in one woman in HY, which we do not consider sufficient for further statistical evaluation. By Student's t-test, we found a statistically significant difference between CG and HY in biochemical parameters TSH ($p < 0.001$) and P ($p = 0.006$) in genotype CC. In CT genotype, there was a statistically significant difference between CG and HY in the ALP

Table 1. *General characteristics, anthropometric parameters and biochemical markers of the studied groups*

Parameters	Control group n = 67 Mean (SD)	Hypothyroid group n = 68 Mean (SD)	p-value
Age (years)	50.90 (12.90)	51.98 (16.20)	0.665
Body height (cm)	163.34 (6.06)	163.56 (7.37)	0.501
Body weight (kg)	72.93 (13.66)	71.80 (15.94)	0.660
Waist circumference (cm)	86.65 (14.08)	89.15 (14.43)	0.311
Hip circumference (cm)	101.69 (8.61)	100.61 (10.49)	0.514
BMI (kg/m^2)	27.08 (5.32)	26.83 (5.55)	0.787
Waist-hip ratio	0.85 (0.11)	0.89 (0.15)	0.059
ALP (μkat/L)	1.16 (0.33)	1.26 (0.40)	0.128
Calcium (mmol/L)	2.27 (0.19)	2.20 (0.25)	0.095
Phosphorus (mmol/L)	0.87 (0.20)	0.97 (0.23)	0.008**
TSH (μIU/ml)	1.94 (1.09)	2.99 (2.17)	< 0.001***
Prealbumin (g/L)	0.24 (0.05)	0.24 (0.04)	0.472

BMI – body mass index; ALP – alkaline phosphatase; TSH – thyroid-stimulating hormone; statistical significance **p < 0.01, ***p < 0.001

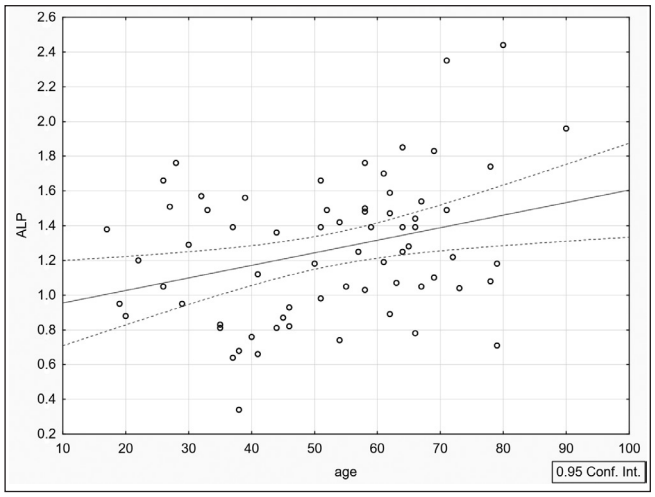

Fig. 1. *Association between age and ALP enzymatic activity in the HY group.*

Equation of a straight line: ALP = 0.88162 + 0.0723* age; coefficient of determination: r = 0.31368^2

parameter (p = 0.030). In multiple comparisons of values, based on Kruskal-Wallis test, we did not find statistically significant differences between CG and HY genotypes in anthropometric parameters and biochemical markers.

DISCUSSION

WHR values are often markers associated with the development of diseases of civilization such as atherosclerosis, dyslipidaemia, hypertension, diabetes mellitus, and others. In addition to the assessment of body fat distribution through WHR index, BMI, urinary and liver function tests, glycaemia, lipid profile, blood pressure, ECG, hormone testing for diseases such as hypothyroidism, hypercortisolism, menstrual disorders, fertility, and insulin resistance can be assessed (12, 13). The authors in a scientific study (14) performed on Indian population compared anthropometric parameters (body height and weight, waist and hip circumference, WHR, and BMI) between control group (n = 100) and hypothyroid women group (n = 100). Comparing the results of our study with this study (14), we found higher values of body height (by 9.56 cm) but lower values of waist circumference (by 1.1 cm), hip circumference (by 2.74 cm), and BMI (by 3.2 kg/m^2) in our HY group. In contrast, in the female control group (CG), we found higher values of body height (by 8.34 cm), body weight (by 14.43 kg), waist circumference (by 8.75 cm), hip circumference (by 6.86 cm), and BMI (by 3.26 kg/m^2). WHR values were higher in CG and HY (by 0.03) than in the aforementioned study. The authors of the study (14) found statistically significant values between the study groups in the parameters of body height (p < 0.05), body weight, waist and hip circumference, BMI, and WHR (p < 0.001). In our study, we did not find statistically significant differences in these studied anthropometric parameters using Student's t-test. When comparing the results of our study with those of the authors (15) who performed a scientific study in Brazilian women, we again found higher WHR index in the CG and HY groups. This finding suggests that WHR may be a risk factor associated with the development of CVD related to the accumulation of body fat in the central part of the body. Relevant findings were that women with hypothyroidism in the study by Savita et al. and Azevêdo et al. (14, 15) and in our study had higher body fat percentage compared to women in the control group. The reason for this difference may be due to variation in the levels of thyroid hormones that regulate cellular metabolic activity. TSH levels were positively correlated with body weight gain in women in a study of Fox et al. (16). In our scientific study, we also found a positive correlation between TSH and body weight (p < 0.05). Scientific study conducted in Greek population focused on observing correlations between anthropometric parameters and thyroid hormones (17). Using Pearson's correlation coefficient, the authors found no statistically significant relationship between TSH and anthropometric parameters. In our scientific study, a positive correlation was found between TSH and body height (r = 0.371), TSH and body weight (r = 0.285) in the group

Table 2. *Genotype and allele frequencies of PAX gene rs104893657 single nucleotide polymorphisms in Slovak women*

Genotype frequencies						
Group	**CC n (%)**	**CT n (%)**	**TT n (%)**	**HWE**	**χ^2**	**p-value**
Control (n = 67)	62 (92.54)	3 (4.48)	2 (2.99)	0.001***	0.477	0.788
Hypothyroid (n = 68)	63 (92.65)	4 (5.88)	1 (1.47)	0.013*		
Allele frequencies						
Group	**C n (%)**	**T n (%)**	**χ^2**	**p-value**	**OR**	**95% CI**
Control	127 (94.78)	7 (5.22)	0.097	0.755	0.837	0.274–2.560
Hypothyroid	130 (95.59)	6 (4.41)				

χ^2 – chi-square test; OR – odds ratio; CI – confidence interval; statistical significance *p < 0.05, ***p < 0.001, HWE – Hardy-Weinberg equilibrium, investigated polymorphism in the given population is not in H-W; we justify this by the fact that between genotype frequencies and theoretically calculated frequencies for a given population group (observed versus expected number) is a statistically significant difference (p less than 0.05).

of women with HY ($p < 0.05$). The relationship between BMI and TSH was studied by Gupta et al. (18) in Indian women with subclinical hypothyroidism. The authors found a positive correlation between TSH and BMI ($p < 0.05$) in CG and HY. In our study, there was no correlation between TSH and BMI parameters in either of the study groups. A very sensitive negative acute phase protein is prealbumin. It is one of the transport proteins of the thyroid hormones T3 and T4. Increased levels of this marker occur in adrenal hyperfunction, hypothyroidism, prednisone treatment, glomerular and tubular proteinuria (19). Its determination is part of several scientific studies in connection with thyroid activity. Henze et al. (20) measured prealbumin levels in women with treated and untreated hypothyroidism, hyperthyroidism and in a control group of women. In all groups, prealbumin levels were within reference ranges. Our mean PREA values were the same in CG (0.24 ± 0.05 g/L) and HY (0.24 ± 0.04 g/L), as well as reported by Henze et al. (20) in their study in the Turkish population. The effect of thyroid hormones on ALP enzymatic activity in hypothyroid women has been studied by Mane and Bhagwat, Prathyusha and Priya, and Yadav et al. (21–23) and others. In our scientific study we found lower values of ALP enzymatic activity in CG and HY compared to other studies (22, 23). Thyroid hormones are needed for the function, growth and development of all organs in the body. They regulate the basal metabolism of all cells of the body, including hepatocytes, and thus modulate the function of the liver. The liver metabolises thyroid hormones and regulates their systemic endocrine effects. Therefore, thyroid disorders can impair liver function and, by contrast, liver disease affects thyroid hormone metabolism and various systemic diseases (24). In our study, we found no correlation of TSH with ALP levels in the group of women with thyroid dysfunction. Proper thyroid function helps in the regulation of calcium in the blood stream and influences the absorption of calcium from the intestine. A scientific study by Susanna et al. (25) was focused on the comparison of thyroid parameters and mineral profile in Indian subjects with hypothyroidism and control group. The authors reported the same mean Ca value in the control and hypothyroid women groups (2.245 mmol/L), mean P value in CG

Table 3. *Biochemical and anthropometric markers in relation to the genotypes of the rs104893657 PAX8 gene polymorphism*

Parameters	**CC**			**CT**		
	CG Mean (SD)	**HY Mean (SD)**	**p-value**	**CG Mean (SD)**	**HY Mean (SD)**	**p-value**
Number	62/67	63/68		3/67	4/68	
Body height (cm)	164.34 (6.20)	163.54 (7.48)	0.444	160.00 (2.00)	163.75 (7.54)	0.449
Body weight (kg)	73.44 (13.94)	71.24 (16.20)	0.419	70.00 (9.17)	77.25 (12.26)	0.432
Waist circumference (cm)	87.11 (14.38)	88.52 (14.60)	0.589	84.83 (9.28)	93.75 (7.53)	0.217
Hip circumference (cm)	101.87 (8.68)	101.35 (10.71)	0.386	102.61 (10.21)	103.38 (8.06)	0.885
BMI (kg/m^2)	27.23 (5.43)	26.62 (5.60)	0.537	27.29 (2.93)	28.95 (5.09)	0.640
Waist-hip ratio	0.85 (0.11)	0.89 (0.15)	0.129	0.84 (0.12)	0.91 (0.02)	0.284
ALP (µkat/l)	1.18 (0.33)	1.26 (0.41)	0.258	0.77 (0.19)	1.32 (0.27)	0.030*
Calcium (mmol/L)	2.26 (0.19)	2.21 (0.26)	0.170	2.22 (0.26)	2.11 (0.12)	0.474
Phosphorus (mmol/lL)	0.87 (0.20)	0.98 (0.24)	0.006**	0.83 (0.09)	0.87 (0.06)	0.542
TSH (µIU/ml)	1.91 (1.06)	2.95 (2.2.20)	< 0.001***	1.40 (0.81)	3.71 (2.14)	0.141
Prealbumin (g/L)	0.24 (0.05)	0.24 (0.04)	0.729	0.27 (0.09)	0.22 (0.04)	0.337

CC and CT – genotypes; CG – control group; HY – group of women with hypothyroidism; ALP – alkaline phosphatase; TSH – thyroid-stimulating hormone; statistical significance *p < 0.05

(0.733 mmol/L), and in HY (1.397 mmol/L). Comparing these results with our study, we found a mean value of Ca (2.27 ± 0.19 mmol/L), P (0.87 ± 0.20 mmol/L) in CG and HY (Ca 2.20 ± 0.25 mmol/L; P 0.97 ± 0.23 mmol/L). Higher phosphorus values were observed in women with HY. Based on statistical evaluation by Student's t-test, we found a statistically significant difference ($p = 0.008$) between CG and HY in this parameter in our study. In addition to its role in thyroid development, the *PAX8* gene is an important regulator of the activation of the expression of specific genes, namely thyroid peroxidase (TPO) and thyroglobulin (TG) (26). The aim of this presented scientific study was to analyse the representation of genotypes and alleles of the rs104893657 polymorphism of the *PAX8* gene in women with hypothyroidism and in a control group of women. To determine whether there is a correlation between the given variant of the *PAX8* gene with the phenotypes (anthropometric parameter, biochemical marker). Alcántara-Ortigoza et al. (27) identified 3 novel intronic variants of the *PAX8* gene and 5 novel exon substitutions in the Mexican population. The identified variant was also a mutation designated as Arg31His (rs104893657) with clinical impact. The allelic frequencies of the novel variants were different in CG and HY. In our study, the frequency of the homozygous genotype for the mutant allele was higher in the HY group (TT = 1.47%) and in the CG group (TT = 2.99%). Heterozygous genotype was present in frequency in HY group (CT = 5.88%) and CG group (CT = 4.48%). The occurrence of the T allele in CG suggests that the women in question have a genetic predisposition to congenital hypothyroidism within the rs104893657 *PAX8* gene. When looking at the genotype versus phenotype association, we found a statistically significant difference between CG and HY in P ($p = 0.006$) and TSH ($p < 0.001$) parameters in the CC genotype. The rs104893657 polymorphism of the *PAX8* gene is poorly studied in association with hypothyroidism. Scientific studies by Vincenzi et al., Komatsu et al., Ramos et al., and Cerqueira et al. (7, 28–30) are mainly focused on the detection of polymorphisms of different genes that are associated with thyroid dysgenesis, congenital hypothyroidism. Our study provides initial information on the frequency representation of genotypes and alleles of the rs104893657 *PAX8* gene polymorphism in the Slovak female population, therefore we consider these results unique.

CONCLUSION

The results of our study provide initial information on the distribution of genotypes and alleles of the rs104893657 variant of the *PAX8* gene in a group of Slovak women with hypothyroidism. An important finding was that the mutant T allele was detected at low frequency in both CG and HY groups of women but the association of the T allele with the onset of hypothyroidism was not confirmed. Highly specialized biochemical markers are the basis for the diagnosis of reduced thyroid function. Within the biochemical markers, we also monitored the specific parameter prealbumin (a carrier of thyroid hormones). According to our results, we can conclude that the PREA value was not affected by hypothyroid state. Elucidation of pathological mechanisms and complementation of laboratory parameters including ultrasonography and molecular genetic screening in the diagnosis of thyroid disorders is of great importance in predictive diagnostics, since these are diseases that affect all populations worldwide with a higher prevalence in women. In the future, we recommend that this issue be further addressed to expand the pool of individuals and to monitor other biochemical markers associated with this disease.

Conflicts of Interest

None declared

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