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Age at diagnosis and gender modify the risk of 9q22 and 14q13 polymorphisms for papillary thyroid carcinoma

Wiek zachorowania i płeć jako czynniki modyfikujące związek polimorfizmów zlokalizowanych na chromosomie 9q22 i 14q13 z rakiem brodawkowatym tarczycy

Dorota Kula[1], Michał Kalemba[1], Zbigniew Puch[1], Joanna Polańska[2], Michał Świerniak[3,4], Dagmara Rusinek[1], Jadwiga-Żebracka-Gala[1], Małgorzata Kowalska[1], Daria Handkiewicz-Junak[1], Monika Kowal[1], Tomasz Tyszkiewicz[1], Ewelina Piasna[1], Agnieszka Czarniecka[5], Agnieszka Pawlaczek[1], Jolanta Krajewska[1], Sylwia Szpak-Ulczok[1], Barbara Jarząb[1]

[1]*Department of Nuclear Medicine and Endocrine Oncology, Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch, Poland*
[2]*Institute of Automatic Control, Silesian University of Technology, Gliwice, Poland*
[3]*Department of Genomic Medicine, Medical University of Warsaw, Poland*
[4]*Centre of New Technologies, CENT, University of Warsaw, Poland*
[5]*Clinic of Oncology and Reconstructive Surgery, Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch, Poland*

Abstract

Introduction: Papillary thyroid cancer (PTC) shows familial occurrence, and some susceptibility single nucleotide polymorphisms (SNPs) have been identified in *FOXE1* and near the *NKX2-1* locus. The aim of our study was to analyse the association of PTC risk with SNPs in *FOXE1* (rs965513, rs1867277, rs1443434) and near the *NKX2-1* locus (rs944289) in a Polish population, and, in the second step, the interaction between SNPs and patient-related factors (age at diagnosis and gender).

Material and methods: A total of 2243 DNA samples from PTC patients and 1160 controls were included in the study. The SNP analysis was performed with the allelic discrimination technique.

Results: There were significant associations of all SNPs with PTC (rs965513 odds ratio [OR] = 1.72, $p = 8 \times 10^{-7}$; rs1867277 OR = 1.59, $p = 1 \times 10^{-6}$; rs1443434 OR = 1.53, $p = 1 \times 10^{-5}$; rs944289 OR = 1.52, $p = 4 \times 10^{-5}$). Logistic regression analysis revealed an increased PTC risk in the interaction of rs944289 with age at diagnosis (OR = 1.01 per year, $p = 6 \times 10^{-4}$) and a decreased PTC risk in the interaction of male gender with the GGT *FOXE1* protective haplotype (OR = 0.69, p = 0.01).

Conclusions: The association between PTC and all analysed SNPs was confirmed. It was also shown that patient-related factors modify the predisposition to PTC by increasing the risk for rs944289 per year of age, and by enhancing the protective effect of the *FOXE1* GGT haplotype in men. **(Endokrynol Pol 2017; 68 (3): 283–289)**

Key words: *SNP; carcinoma papillare; age at diagnosis; gender*

Streszczenie

Wstęp: Brodawkowaty rak tarczycy należy do grupy nowotworów litych, w których uwarunkowanie genetyczne ogrywa istotną rolę. Geny odpowiedzialne za predyspozycje do raka brodawkowatego nie są dobrze znane, choć polimorfizmy rs965513 i rs944289 obecnie są uznanymi czynnikami ryzyka. Celem pracy była analiza związku polimorfizmów znajdujących się w 9q22 w locus genu *FOXE1* (rs965513, rs1867277, rs1443434) oraz w 14q13 w pobliżu genu *NKX2-1* (rs944289) z rakiem brodawkowatym tarczycy oraz ocena wpływu czynników zależnych od pacjenta (wieku zachorowania i płci).

Materiał i metody. Materiał obejmował 2243 próbek DNA izolowanych z limfocytów krwi obwodowej pacjentów z rakiem brodawkowatym i 1160 próbek DNA pochodzących od osób zdrowych, stanowiących grupę kontrolną (liczba analizowanych próbek różniła się w zależności od polimorfizmu). Badania wykonano w aparacie 7900HT Fast Real-Time PCR System firmy Applied Biosystems techniką dyskryminacji alleli.

Wyniki. Znamienny związek z rakiem brodawkowatym wykazywały wszystkie analizowane polimorfizmy (dla rs965513 wartość OR wynosiła 1,72, $p = 8 \times 10^{-7}$; dla rs1867277 OR = 1,58, $p = 1 \times 10^{-6}$; dla rs1443434 OR = 1,53, $p = 1 \times 10^{-5}$; rs944289 OR = 1,52, $p = 4 \times 10^{-5}$). Analiza regresji logistycznej wykazała wzrost ryzyka raka brodawkowatego wraz z wiekiem dla polimorfizmu rs944289 (OR = 1.01 na rok, $p = 6 \times 10^{-4}$) oraz obniżenie ryzyka zachorowania dla haplotypu GGT genu *FOXE1* u mężczyzn (OR = 0,69, p = 0,01).

Wnioski. Potwierdzony został związek badanych polimorfizmów z rakiem brodawkowatym tarczycy w populacji polskiej. Wykazano modyfikujący wpływ wieku zachorowania i płci męskiej na ryzyko zachorowania uwarunkowane genetycznie. **(Endokrynol Pol 2017; 68 (3): 283–289)**

Słowa kluczowe: *polimorfizm; rak brodawkowaty tarczycy; wiek zachorowania; płeć*

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Dorota Kula M.D., Department of Nuclear Medicine and Endocrine Oncology, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch, Wybrzeże Armii Krajowej 15, 44–101 Gliwice, Poland, phone: +48 32 278 9720, fax: +48 32 278 9457, e-mail: dorota.kula@io.gliwice.pl

Introduction

Papillary thyroid cancer (PTC) is the most frequently diagnosed differentiated thyroid cancer (DTC) [1, 2]. Genetic predisposition to PTC is the highest of all cancers not displaying Mendelian inheritance [3–6]. This predisposition is expected to be multigenetic, with interactions among genes and environmental factors determining individual susceptibility [2, 4]; however, the genes responsible for PTC are poorly known. To date, genetic studies have identified some single nucleotide polymorphisms (SNPs) associated with DTCs or PTC, although only some of them have been validated [1, 7]. In 2009 Gudmundsson et al. identified SNPs associated with PTC: rs965513, located upstream of the *FOXE1* gene on chromosome 9q22.33; and rs944289, located upstream of the *NKX-2* gene on chromosome 14q13.3 [5]. At the same time, Landa et al. demonstrated an association between PTC and another SNP located in the 5-untranslated region of the *FOXE1* — rs1867277 [4]. However, to date, only a few studies have been performed to assess the association of these SNPs with some clinical features of PTC [5, 8, 9]. To our knowledge, no study has analysed the mutual interactions between identified SNPs and their association with patient-related factors, like gender and age at PTC diagnosis.

In the present study, we first analysed the association of SNPs in the *FOXE1* locus (rs965513, rs1867277, rs1443434) and near the *NKX2-1* gene (rs944289) with PTC. Next, we evaluated the interactions between these SNPs and their associations with gender and age at PTC diagnosis.

Material and methods

Samples and subjects

A total of 2243 DNA samples derived from PTC patients and 1160 samples from controls were analysed. All subjects were Caucasians of Polish origin. There were 1925 (85.8%) women in the PTC group and 949 (81.8%) women in the control group. The median age at diagnosis of PTC was 48 years (first–third quartile: 37–57 years). The median age for the control group was 45 years (first–third quartile: 33–54 years). The number of samples analysed differed depending on the SNP and DNA availability (Table I). Only samples with all SNPs evaluated were included in the linkage disequilibrium (LD), haplotype frequency, and logistic regression analyses (Table II, III, IV, and VI, respectively).

PTC cases were recruited among thyroid cancer patients from all over Poland, who were referred for treatment to the Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch. Data on thyroid cancer histopathology, patient gender, and age at diagnosis were extracted from medical records. Control subjects were recruited among consenting volunteers (from the population in general, from workers of the Institute of Oncology, and the persons accompanying the patients attending the Institute, not related to the patients) and were derived from the same populations as the cases. In all control subjects, thyroid cancer was excluded by anamnesis and thyroid ultrasound. The study was performed with approval from the Local Bioethics Committee, and written, informed consent was obtained from all participants.

DNA extraction and SNP analysis

DNA was isolated from the whole blood. Two main DNA extraction methods were used, i.e., the salting-out method and the anion exchange membrane column separation method (Genomic Maxi AX Blood; A&A Biotechnology, Gdynia, Poland), performed according to the manufacturer's protocols. Initial screening of DNA purity was determined based on evaluation of the optical density ratio at 260/280 nm.

The SNP analysis with the allelic discrimination technique was performed on 384-well plates (Applied Biosystems, Foster City, CA, USA) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a final volume 5.1 μl per sample, containing 0.125 μl of 40X TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA), 2.5 μl of 2X TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA), and 2.5 μl of DNA (at concentrations of 3 ng/μl).

Statistical analysis

STATISTICA 6 software was used to evaluate the genotypes frequency. The odds ratios (ORs) were calculated with their 95% confidence intervals (CI). The deviation from Hardy-Weinberg equilibrium for each polymorphism was verified. Due to multiple testing, the Bonferroni correction was applied to p-values resulting from χ^2 tests. Haplotype frequencies were estimated with the use of the EM algorithm. Logistic regression analysis was performed to model the effects of analysed SNPs and *FOXE1* haplotypes, corrected for age at diagnosis and gender. For the logistic regression, the Akaike's Information Criterion-corrected (AICc) value was used to evaluate models. A p-value less than 0.05 was considered to be statistically significant.

Results

Association of SNPs with risk of PTC

All analysed SPNs were in Hardy-Weinberg equilibrium both in the case and control groups. The genotype distributions, allele frequencies, and ORs for all SNPs

Table I. *Genotype distribution of analysed SNP among PTC patients and controls*

Tabela I. *Rozkład genotypów badanych polimorfizmów w grupie chorych z rakiem brodawkowatym i w grupie kontrolnej*

	Sample	Median Age (Q1–Q3)	Females (%)	Males (%)	N	Genotype			Allele frequency		OR (95% CI)	p-value	Bonferroni-corrected p-value
rs965513* risk allele: A						**AA (%)**	**AG (%)**	**GG (%)**	**A**	**G**			
	PTC	47 (37–56)	717 (86.1)	116 (13.9)	833	196 (23.5)	406 (48.7)	231 (27.7)	0.48	0.52	1.72 (1.40–2.11)	2×10^{-7}	8×10^{-7}
	Controls	44 (33–54)	699 (82.7)	146 (17.3)	845	125 (14.8)	384 (45.4)	336 (39.8)	0.38	0.62			
rs1867277* risk allele: A						**AA (%)**	**AG (%)**	**GG (%)**	**A**	**G**			
	PTC	48 (37–57)	1481 (85.9)	243 (14.1)	1724	405 (23.5)	879 (51.0)	440 (25.5)	0.49	0.51	1.59 (1.33–1.89)	3×10^{-7}	1×10^{-6}
	Controls	44 (33–54)	721 (83.0)	148 (17.0)	869	158 (18.2)	405 (46.6)	306 (35.2)	0.41	0.59			
rs1443434* risk allele: G						**GG (%)**	**GT (%)**	**TT (%)**	**G**	**T**			
	PTC	48 (37–57)	1523 (85.6)	256 (14.4)	1779	413 (23.2)	909 (51.1)	457 (25.7)	0.49	0.51	1.53 (1.28–1.84)	3×10^{-6}	1×10^{-5}
	Controls	44 (33.5–54)	651 (82.6)	137 (17.4)	788	145 (18.4)	370 (47.0)	273 (34.6)	0.42	0.58			
rs944289* risk allele: T						**TT (%)**	**TC (%)**	**CC (%)**	**T**	**C**			
	PTC	48 (37–57)	1925 (85.8)	318 (14.2)	2243	877 (39.1)	1049 (46.8)	317 (14.1)	0.63	0.37	1.52 (1.26–1.83)	1×10^{-5}	4×10^{-5}
	Controls	45 (33–54)	949 (81.8)	211 (18.2)	1160	364 (31.4)	564 (48.6)	232 (20.0)	0.56	0.44			

Table II. *Linkage disequilibrium for SNPs located in the FOXE1 locus*

Tabela II. *Analiza nierównowagi sprzężeń dla polimorfizmów zlokalizowanych w locus genu FOXE1*

	PTC N = 663			Controls N = 590		
	D′	**χ^2**	**p-value**	**D′**	**χ^2**	**p-value**
rs965513:G & rs1867277:G	0.5371	771.3	$< 10^{-6}$	0.7668	1456.5	$< 10^{-6}$
rs965513:G & rs1443434:T	0.7889	767.8	$< 10^{-6}$	0.7877	1523.6	$< 10^{-6}$
rs1867277:G & rs1443434:T	0.9971	1431.8	$< 10^{-6}$	0.9692	2571.6	$< 10^{-6}$

D′ — normalised coefficient of linkage disequilibrium; χ^2 — the value of chi-square statistic; SNP — single nucleotide polymorphism; PTC — papillary thyroid carcinoma

are presented in Table 1. A significant association for all analysed SNPs was observed regarding all models of inheritance: genotypic, allelic, dominant, and recessive (data not shown). Only OR values for the dominant model are included in Table I: 1.72, 1.59, 1.53, 1.52 for rs965513, rs1867277, rs1443434, and rs944289, respectively; all of them significant after Bonferroni correction.

SNPs located in the *FOXE1* locus (rs965513, rs1867277, rs1443434) were in LD (pairwise D′ ranged from 0.5371 to 0.9971 with p values $< 10^{-6}$) (Table II). AAG *FOXE1* haplotype was more frequent in PTC cases than in controls (40.5% *vs.* 32.5%), and its presence was significantly associated with PTC risk (OR= 1.42, $p = 1.1 \times 10^{-5}$) (Table III). By contrast, there was a lower frequency of GGT haplotype in PTC patients than in controls (43.3% versus 53.7%), and this haplotype was significantly associated with a reduced risk of PTC (OR = 0.66, $p = 8 \times 10^{-8}$) (Table IV).

Table III. *Haplotype frequency of SNPs located in the FOXE1 locus*
Tabela III. *Częstość haplotypów polimorfizmów zlokalizowanych w locus genu FOXE1*

SNP (*FOXE1* locus)			Haplotype frequency			
rs965513 allele	rs1867277 allele	rs1443434 allele	PTC N = 663		Controls N = 718	
G	G	T	0.433026	43.3%	0.536929	53.7%
A	A	G	0.405090	40.5%	0.325230	32.5%
G	A	G	0.071972	7.2%	0.087027	8.7%
A	G	T	0.053840	5.4%	0.050119	5.0%
A	A	T	0.002322	0.2%	0.000696	0.1%
G	A	T	0.013075	1.3%		
G	G	G	0.005306	0.5%		
A	G	G	0.015370	1.5%		

SNP — single nucleotide polymorphism; PTC — papillary thyroid carcinoma

Table IV. *OR values for the most frequent FOXE1 SNP haplotypes*
Tabela IV. *Wartości OR dla najczęściej występujących haplotypów polimorfizmów genu FOXE1*

		rs965513	rs1867277	rs1443434	N	OR (95% CI)	p-value
Risk haplotype	PTC	A	A	G	544/1326 (41%)	1.42 (1.21–1.65)	1.1×10^{-5}
	Controls	A	A	G	473/1436 (33%)		
Protective haplotype	PTC	G	G	T	581/1326 (43%)	0.66 (0.57–0.77)	8.0×10^{-8}
	Controls	G	G	T	777/1436 (54%)		

PTC — papillary thyroid carcinoma; OR — odds ratio; CI — confidence interval; SNP — single nucleotide polymorphism

Association of SNPs with age at diagnosis and gender

The PTC patients were divided into two subgroups with the reference to the age at diagnosis: ≤ 45 years, and > 45 years. (Table V). Differences were observed only for rs965513, but they were not significant after Bonferroni correction. We did not observe any difference in genotype distribution between women and men in the univariate analysis (data not shown).

Logistic regression

In the first step of the analysis, evaluation of individual effects of age and gender was performed - both of them were significantly associated with PTC risk (p = 0.0032 and 0.0375; AICc = 1907.5 and 1911.9, respectively). Next, models of inheritance were evaluated. For all analysed SNPs and FOXE1 locus haplotypes, the additive model emerged with the lowest AICc value. Finally, based on the AICc, rs1867277, rs944289, the GGT protective haplotype, age at diagnosis, and gender were included into the final regression analysis (Table VI), which showed an increased risk of PTC for rs944289 (OR = 2.65, p = 1.47 × 10-6) and a decreased risk for the GGT haplotype (OR = 0.54, p = 3 × 10-4). Moreover, analysis of interactions revealed an enhanced risk for rs944289 per year of age (OR = 1.014, p = 6 × 10-4) and an enhanced protective effect of the GGT haplotype in men (OR = 0.699, p = 0.012).

Discussion

We observed the association of the rs965513, rs1867277, rs1443434, and rs944289 SNPs with PTC, and two of them (rs1867277 and rs1443434) have not yet been analysed in a Polish population. The association of rs1867277 with PTC was first described by Landa et al., based on a case-control study [4], while rs1443434 b was first described by Gudmundsson et al. in a GWAS [5] — the results were similar to those obtained in our study. The association of rs965513 and rs944289 with PTC was analysed by Liyanarachchi et al. in cohorts from Poland and the United States [2], with results that are in accordance with our study.

The association of rs965513, rs1867277, rs944289, and rs1443434 with PTC has been confirmed in different populations in recent years [8–17]. Moreover, four meta-analyses concerning the roles of rs965513, rs1867277, and rs944289 in PTC have been published: the association of rs965513 has been confirmed among both Caucasians and Asians, rs1867277 SNP was ana-

Table V. *Associations of analysed polymorphisms with age at PTC diagnosis*
Tabela V. *Związek badanych polimorfizmów z wiekiem diagnozy raka brodawkowatego*

	Age group (years)	N	Genotype			OR (95% CI)	p-value	Bonferroni-corrected p-value
rs965513 risk allele: A			**AA (%)**	**AG (%)**	**GG (%)**			
	≤ 45	383	100 (26.1)	192 (50.1)	91 (23.8)	1.45 (1.06–1.99)	0.018	0.072
	> 45	450	96 (21.3)	214 (47.6)	140 (31.1)			
rs1867277 risk allele: A			**AA (%)**	**AG (%)**	**GG (%)**			
	≤ 45	766	158 (20.6)	415 (54.2)	193 (25.2)	1.03 (0.83–1.28)	0.78	ns
	> 45	958	247 (25.8)	464 (48.4)	247 (25.8)			
rs1443434 risk allele: G			**GG (%)**	**GT (%)**	**TT (%)**			
	≤ 45	790	163 (20.6)	423 (53.6)	204 (25.8)	0.99 (0.80–1.22)	0.91	ns
	> 45	989	250 (25.3)	486 (49.1)	253 (25.6)			
rs944289 risk allele: T			**TT (%)**	**TC (%)**	**CC (%)**			
	≤ 45	995	389 (39.1)	470 (47.2)	136 (13.7)	1.07 (0.84–1.36)	0.57	ns
	> 45	1248	488 (39.1)	579 (46.4)	181 (14.5)			

ns — not significant; PTC — papillary thyroid carcinoma; OR — odds ratio for dominant model of inheritance; CI — confidence interval

Table VI. *Logistic regression analysis*
Tabela VI. *Analiza regresji logistycznej*

	Coefficients Estimate	Standard Error	OR (95% CI)	p-value
Subjects: 663 PTC cases, 718 controls				
Main effects				
Additive, rs1867277	–0.26438	0.17051	0.7677 (0.5496–1.0723)	0.121015
Additive, rs944289	0.97571	0.20262	2.6532 (1.7835–3.9463)	1.47×10^{-6}
Additive, protective haplotype GGT	–0.62066	0.17172	0.5376 (0.3840–0.7527	0.000301
Interactions				
Age (per year): rs944289	0.01367	0.00399	1.0138 (1.0059–1.0217)	0.000610
Gender (male): additive, protective haplotype GGT	–0.35705	0.14209	0.6997 (0.5296–0.9245)	0.011979
AICc	1856.16			
Residual deviance	1844.1			

AICc — Akaike's Information Criteria-corrected; OR — odds ratio; CI — confidence interval

lysed and confirmed in Caucasians [18–20], whereas rs944289 was associated with PTC in Asians, Europeans, and Americans [21] .

The SNPs rs1867277 and rs965513 in the *FOXE1* locus are in moderate pairwise LD in Europeans [12, 13]; however, these two SNPs were found to be more strongly correlated in a Belarusian population [17]. LD between rs965513 and rs1867277 is significantly weaker in populations of African or Asian ancestry [20], which is not surprising, since the LD block structure may be

different in individuals of different ancestry [22]. In our study we observed LD between rs965513, rs1867277, and rs1443434; however, that between rs1867277 and rs1443434 was the strongest. Our analysis of rs965513/ /rs1867277/rs1443434 *FOXE1* haplotypes showed an increased risk of PTC for the AAG haplotype and a reduced risk for the GGT haplotype. Similar results were obtained by Jones et al. [13], who showed that carriers of haplotypes consisting of both risk alleles of rs965513 and rs1867277 in a population from the United Kingdom had the highest risk of developing thyroid cancer [13]. A significant association also was observed for a haplotype in 9q22 consisting of a *FOXE1* polymorphism: rs965513/ rs10759944/rs1867277 in thyroid cancer families [14].

In our study, some differences in the genotype distributions of rs965513 were observed between younger (diagnosed at ≤ 45 years of age) and older (diagnosed at > 45 years of age) patients, although these differences were not significant after Bonferroni correction. We did not observe differences in genotype distribution between women and men in the univariate analysis (data not shown). To integrate the results concerning the genotype distribution of rs965513, rs1867277, rs1443434, and rs944289 and their association with patient-related factors (age at PTC diagnosis and gender), we performed a logistic regression analysis. Multivariate analysis showed an increased risk of PTC for rs944289. A decreased risk was observed for the GGT *FOXE1* haplotype. Moreover, analysis of interactions revealed that risk for rs944289 was increased per year of age, and male gender enhanced the protective effect of the *FOXE1* GGT haplotype.

Only a few studies have been performed to assess the association of the analysed SNPs with PTC clinical features [5, 8, 9, 16]. To our knowledge, only one previous study has evaluated the association between age at diagnosis and genetic predisposition, which showed a higher frequency of rs965513 A allele carriers among PTC patients diagnosed at a younger age [5]. Furthermore, only one study has analysed the differences between genders, which showed that rs965513 was significantly associated with PTC among both women and men [8].

The female-to-male ratio of thyroid cancer in European patients is 2.2 [23], and these sex differences in thyroid cancer occurrence have been attributed to different endogenous hormone profiles between the genders. The protective hormonal effects on the development of thyroid cancer in females have been reported, but the mechanism remains unclear [24]. However, opposite effects were recently observed in rats. Fortunato et al. showed that a redox imbalance elicited by oestrogen could be involved in the sex differences found in the prevalence of thyroid dysfunctions [25]. They observed sexual dimorphism in the thyrocyte redox balance, which was characterised by increased hydrogen peroxide production due to higher NOX4 and Poldip2 gene expression and weakened enzymatic antioxidant defence in the thyroids of adult female rats compared with male rats [25]. These findings could lead to the hypothesis that the same genetic background has a different effect in males and females because of different hormone profiles, which would explain the observed enhancement of the protective effect of the *FOXE1* GGT haplotype in men in our study. Thus, the presence of the protective haplotype of the *FOXE1* gene, a thyroid transcription factor gene involved in thyroid peroxidase (TPO) transcription [4, 5], might only reveal its protective effect in males and not females because of the interacting effects of oestrogens. Oestrogens have a general stimulatory effect on thyroid function, at least in rats, by increasing iodide uptake and TPO activity, as well as H_2O_2 production [25], which, as a reactive oxygen species, causes DNA damage, which could lead to the higher frequency of thyroid cancer development in women.

Conclusions

We have confirmed the association of PTC with SNPs in the *FOXE1* locus on chromosome region 9q22.33 (rs965513, rs1867277, rs1443434) and one SNP (rs944289) near the *NKX2-1* gene on chromosome 14q13.3 in a Polish population. The importance of *FOXE1* haplotypes was demonstrated in our population. Logistic regression revealed interesting new interactions: the risk of PTC in carriers of the rs944289 SNP was increased per year of age, and a stronger protective effect of the GGT *FOXE1* haplotype was observed in men. Thus, patient-related factors appear to modify the genetic predisposition to PTC, at least in our population. To our knowledge, this is the first study to evaluate these four SNPs and their associations with patient-related factors in the same patient set.

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