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The *FOXE1* locus is a major genetic determinant for radiation-related thyroid carcinoma in Chernobyl

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Papillary thyroid cancer (PTC) among individuals exposed to radioactive iodine in their childhood or adolescence is a major internationally recognized health consequence of the Chernobyl accident. To identify genetic determinants affecting individual susceptibility to radiation-related PTC, we conducted a genome-wide association study employing Belarusian patients with PTC aged 0–18 years at the time of accident and age-matched Belarusian control subjects. Two series of genome scans were performed using independent sample sets, and association with radiation-related PTC was evaluated. Meta-analysis by the Mantel–Haenszel method combining the two studies identified four SNPs at chromosome 9q22.33 showing significant associations with the disease (Mantel–Haenszel *P*: mhp = 1.7×10^{-9} to 4.9×10^{-9}). The association was further reinforced by a validation analysis using one of these SNP markers, rs965513, with a new set of samples (overall mhp = 4.8×10^{-12}, OR = 1.65, 95% CI: 1.43–1.91). Rs965513 is located 57-kb upstream to *FOXE1*, a thyroid-specific transcription factor with pivotal roles in thyroid morphogenesis and was recently reported as the strongest genetic risk marker of sporadic PTC in European populations. Of interest, no association was obtained between radiation-related PTC and rs944289 (mhp = 0.17) at 14p13.3 which showed the second strongest association with sporadic PTC in Europeans. These results show that the complex pathway underlying the pathogenesis may be partly shared by the two etiological forms of PTC, but their genetic components do not completely overlap each other, suggesting the presence of other unknown etiology-specific genetic determinants in radiation-related PTC.

INTRODUCTION

The Chernobyl accident in April 1986 led to radioactive contamination of vast territories in Belarus, Ukraine and Russia. Millions of residents were exposed to a wide spectrum of radionuclides of which ^{131}I was the major dose-forming isotope for the thyroid. A sharp increase in thyroid cancer incidence among those exposed in childhood or adolescence has

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been reported since the early 1990s. Its specific temporal and geographic distribution was suggestive of a common causative event in the development of the malignancy (1,2), which was later proved to be internal exposure to ^{131}I through its incorporation into food chains of pastured cows and further consumption of fresh milk (3). In 2002, the number of diagnosed thyroid cancers in the three most affected countries approached 5000 of which an estimate of 75% could be attributed to Chernobyl radiation (2,4).

Among the variety of histological types of thyroid cancer, only papillary thyroid carcinoma (PTC) displays evident radiation dose–response and accounts for ~95% cases in the Chernobyl aftermath (3,5,6). Radiation is the only known environmental risk factor for PTC seen both after external exposure (7) and internal irradiation (5). The risk for thyroid cancer in the individuals exposed to radiation at young age remains elevated throughout their lifespan. Although a role of predisposing factors commonly associated with sporadic PTC to the female sex is less relevant in cases of radiation-related PTC, a female to male ratio of 1.6 to 1 has been reported (8). Furthermore, radiation-related PTC is also variable in terms of the duration period of latency, the earliest of which is reported to be 4 years (1). It also remains unclear why, notwithstanding the appreciably comparable thyroid radiation doses in Chernobyl PTC patients and in healthy individuals of the same age and of the same settlements (3,9,10), thyroid malignancy develops only in a small fraction of those exposed. Thus, while radiation dose and young age at exposure are well-established risk factors for PTC, observations are suggestive of an existence of genetic factors and complex gene–environment interactions that may modulate individual radiation sensitivity and susceptibility to radiation-related PTC.

In order to identify genetic determinants that modify individual predisposition to radiation-related thyroid malignancy, we conducted a genome-wide association (GWA) study. Two series of genome scans were performed using two independent sample sets consisting of childhood PTC patients of Belarus and control subjects, followed by a validation study using a third set of case and control samples. A total of 667 patients diagnosed for PTC in 1989–2009, and 827 age-matched controls from the same regions were recruited, comprising the largest collection of patients analyzed to date. In addition, genome scan results of 448 Russian DNA samples were also included as general population controls.

RESULTS

GWA study

In the initial genome scan (termed as Study 1), a total of 532 024 autosomal SNP markers of 187 PTC patients and 172 controls were chosen for a case–control association study after quality control of the genotyping results (Table 1). The average call rates per SNP marker and per DNA sample were 0.999 and 0.999, respectively. No strong deviation of inflation factor was observed between the case and control groups (genomic control inflation factor $\lambda = 1.08$, Supplementary Material, Fig. S1a). A statistical analysis comparing genotype distributions did not find SNP markers that showed genome-wide significance. In the subsequent genome scan (termed as Study 2), 214 cases were examined in the association analysis after quality control, and genotype distributions of 509 610 SNP markers were compared with those of 448 Russian population controls. In Study 2, the average call rates per SNP marker and per DNA sample were 0.998 and 0.980, respectively. A slight inflation of genomic control λ-value was observed between the case and control groups (genomic control inflation factor $\lambda = 1.14$, Supplementary Material, Figs S1b and S2), which is most likely due to within-Russia substructures in the Russian population controls. Again, there were no SNP markers that showed genome-wide significance.

A meta-analysis was undertaken through integration of the genotypes obtained in Study 1 and Study 2. Association with radiation-related PTC was evaluated using the Mantel–Haenszel method for 506 840 SNP markers that passed quality control in both studies. The distribution of the mhp-values along the chromosomes is shown in Figure 1. A slight inflation of λ-value was observed between case and control ($\lambda = 1.11$, Supplementary Material, Fig. S1c). A cluster of four SNPs at chromosome 9q22.23 showed genome-wide significance ($P < 5.0 \times 10^{-8}$), namely, rs925489, rs7850258, rs965513 and rs10759944 with meta-analysis P-values of 1.7×10^{-9}, 1.7×10^{-9}, 4.9×10^{-9} and 3.5×10^{-9}, respectively (Fig. 2 and Table 2). These markers are in strong linkage disequilibrium (LD) to each other (pairwise $D' > 0.999$, $r^2 > 0.999$). Although there were no neighboring SNPs showing stronger signals (Supplementary Material, Table S1), nine other markers at the same chromosomal locus showed suggestive association signals ($\text{mhp} = 5.2 \times 10^{-4}$ to 1.4×10^{-6}) (Table 2). In addition, we examined the association by pooling genotypes obtained in Studies 1 and 2. After correction for population stratification using Eigenstrat as well as for residual inflation by the genomic control method, all four markers that showed genome-wide significance in the meta-analysis were slightly below the level of genome-wide significance (rs7850258: $P = 1.5 \times 10^{-7}$, rs925489: $P = 1.5 \times 10^{-7}$, rs10759944: $P = 2.4 \times 10^{-7}$, rs965513: $P = 3.2 \times 10^{-7}$).

SNP markers located on the X chromosome were tested for association in a separate analysis. Cases and controls were sub-grouped into males and females and association analysis was carried out. As a result, none of the markers showed genome-wide significance ($\text{mhp} > 3.6 \times 10^{-5}$ for males, $\text{mhp} > 1.6 \times 10^{-5}$ for females).

Validation study

The 425-kb region between rs4742698 and rs4618817 encompassing these markers was evaluated for LD structure with the genotyping results of Study 1 and Study 2. Three LD blocks were identified: block A between rs4742698 and rs16924042, block B between rs1512261 and rs10818094 and block C between rs7871887 and rs4618817. All of the four most significant markers are in block B (Fig. 2). There are eight genes that have been localized in the vicinity of these SNPs: *TMOD1* (Entrez Gene ID: 7111), *C9orf97* (ID: 158427), *NCBP1* (ID: 4686), *XPA* (ID: 7507), *KRT18P13* (ID: 392371), *FOXE1* (ID: 2304), *C9orf156* (ID: 51531) and

Table 1. Specification of the DNA samples used for the study

Study	Sample set	Classification	Number	Age at exposure Range	Age at exposure Mean ± SD	Age at diagnosis Range	Age at diagnosis Mean ± SD
Study 1	PTC1	Cases	187	0 – 17	3.0 ± 3.8	3 – 20	10.0 ± 4.1
	CTR1	Controls	172	0 – 17	1.5 ± 2.8	—	—
Study 2	PTC2	Cases	214	0 – 17	5.8 ± 5.2	2 – 22	13.9 ± 5.5
	CTR2	Controls[a]	448	—	—	—	—
Study 3	PTC3	Cases	259	0 – 18	6.8 ± 5.5	3 – 22	16.5 ± 4.4
	CTR3	Controls	648	0 – 26	6.2 ± 5.9	—	—

[a]Russian population controls from other genetic studies.

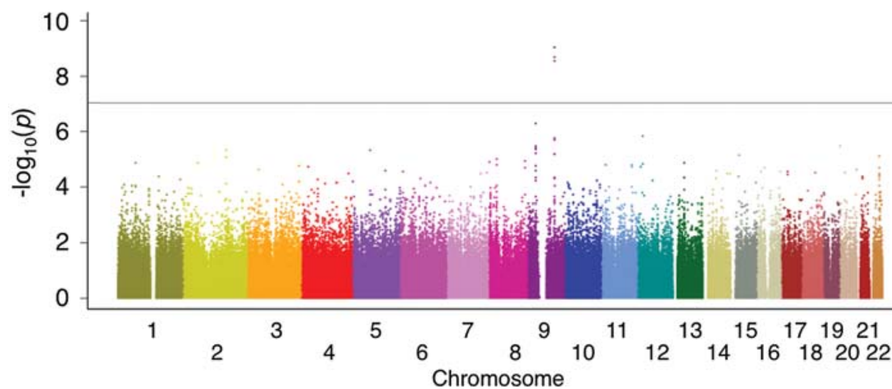

Figure 1. Manhattan plot of the combined GWAS results for Studies 1 and 2. *P*-values calculated by the Trend χ^2 test for 506 840 autosomal SNPs are plotted in $-\log_{10}$ scale with respect to their chromosomal positions. The horizontal line indicates Bonferroni-adjusted $P = 9.6 \times 10^{-8}$.

HEMGN (ID: 55363), but none of these genes reside in block B. Seven out of the nine markers showing suggestive association signals are located at either 5′ or 3′ flanking region of the *FOXE1* gene in block C. In addition, an imputation analysis was performed for SNP markers in blocks A, B and C using genotypes of International HapMap Project as reference. We identified three additional SNPs in block B, namely rs7030280, rs10983700 and rs1588635, located approximately 9–11 kb centromeric to rs925489, showing similar levels of association (imputed $P = 2.8 \times 10^{-9}$ for rs7030280 and rs10983700, imputed $P = 3.7 \times 10^{-9}$ for rs1588635) (Supplementary Material, Table S2). No other SNP markers in block A or block C reached genome-wide significance.

This region at 9q22.23 containing the *FOXE1* (or *TTF2*) gene which encodes a thyroid-specific transcription factor was recently identified as a chromosomal locus strongly associated with predisposition to sporadic thyroid cancer in an Icelandic study (11). Among the seven SNPs showing significant associations ($P < 2.8 \times 10^{-9}$) in the Icelandic sporadic PTC patients, rs965513 was strongest ($P = 6.8 \times 10^{-20}$, OR = 1.77) (Table 2). We therefore selected rs965513 located ~57 kb upstream to *FOXE1* for further genotyping by Taqman using an independent sample set (termed as Study 3) of 259 cases and 648 controls (Table 1). The strong association ($P = 2.0 \times 10^{-4}$) was reproduced and was further reinforced when the genotypes of the three studies were combined for meta-analysis (mhp = 4.8×10^{-12}, OR = 1.65, 95% CI: 1.43–1.91).

Very recently, another genetic study focusing on 97 candidate genes mediating thyroid carcinogenesis identified rs1867277 in the 5′-UTR of *FOXE1* as a genetic determinant for sporadic PTC ($P = 5.9 \times 10^{-9}$, OR = 1.49, 95% CI: 1.30–1.70) (12). Since rs1867277 was not examined in our study, we designed a Taqman probe and genotyped 660 PTC cases (PTC1, PTC2 and PTC3) and 820 Belarusian controls (CTR1 and CTR3) (Table 1). A significant association was obtained with a *P*-value of 4.5×10^{-7} and OR of 1.48 (95% CI: 1.27–1.71).

Genotyping of rs944289 at chromosome 14q13.3

Rs944289 at chromosome 14q13.3 showed the second strongest association with sporadic PTC in the Icelandic population ($P = 2.5 \times 10^{-8}$, OR = 1.44, 95% CI: 1.26–1.63) (11). This SNP is located in a 249-kb LD region which does not contain any known genes, but it lies close to *TTF1* (ID: 7080), another thyroid-specific transcription factor gene. We investigated whether rs944289 showed significant association in our genome scan results. Of our interest, it failed to show any association with radiation-related PTC ($P = 0.23$ in Study 1, $P = 0.43$ in Study 2 and mhp = 0.17 by meta-analysis) (Table 2).

Correlation between rs965513 genotypes and disease latency

It is considered that thyroid cancer requires an induction and latency period of at least 10 years after exposure to ionizing radiation (13). We divided the 660 case samples into two groups depending on the date of diagnosis being either within, or more than, 10 years since radiocontamination.

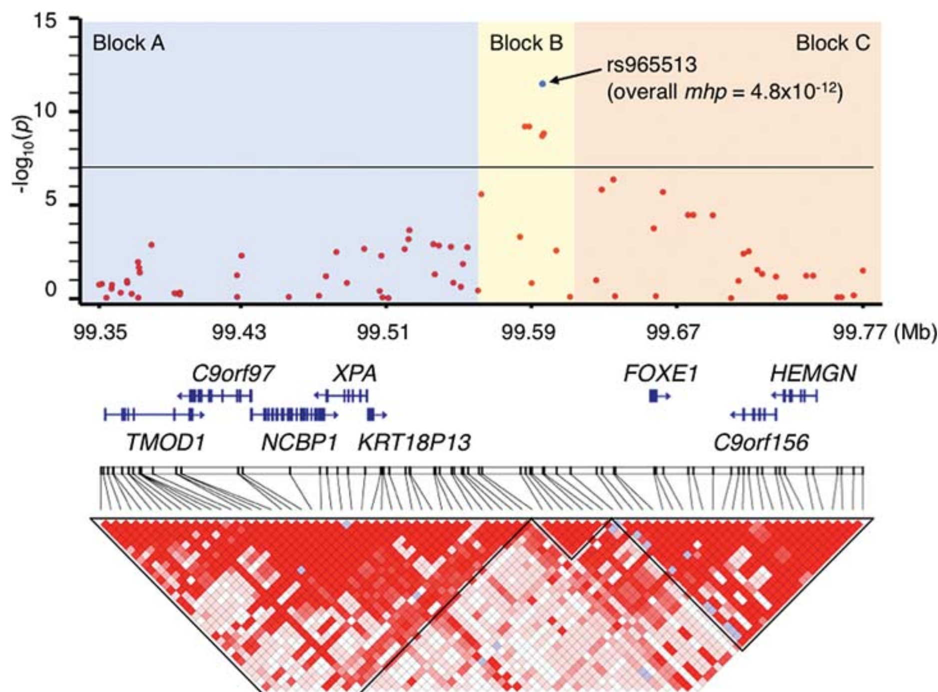

Figure 2. A schematic organization of the human *FOXE1* locus at 9q22.23 with the genome scanning results. Mhp-values calculated by the Trend χ^2 test in $-\log_{10}$ scale were plotted in red circles for SNPs located in the 425-kb region between rs4742698 and rs4618817 at chromosome 9q22.23. The blue circle indicates mhp-value of rs965513 by meta-analysis using the combined results of Study 1 to Study 3. The structure and orientation of eight genes in the region were shown below the plots with their transcriptional orientations according to NCBI Reference Sequence Build 36.3. LD blocks were generated according to pairwise LD estimates of the SNPs located within the region using the genome scan results of Study 1 and Study 2.

For the early-onset group, there were 178 patients aged 3–25 years (mean age ± SD: 11.2 ± 4.3 years) who were diagnosed within the first 10 years (before 1997), with latency of 7.0 ± 1.9 years. For the late-onset group, there were 482 samples aged 10–39 years (22.9 ± 7.5 years) who were diagnosed after 1997, with latency of 16.4 ± 3.8 years. Looking at the results for rs965513, there was a much stronger association observed for the early-onset cases ($P = 2.0 \times 10^{-9}$, OR = 1.97, 95% CI: 1.58–2.47) than the late-onset cases ($P = 6.0 \times 10^{-8}$, OR = 1.52, 95% CI: 1.31–1.77) when compared with 1268 controls (CTR1 to CTR3). However, there was no statistical significance to prove the stronger impact of rs965513 on the early-onset of PTC (p-heterogeneity = 0.063).

DISCUSSION

In this study, we have undertaken a GWA study of radiation-related PTC employing Belarusian patients and control subjects. We identified four markers in strong LD at chromosome 9q22.23 that were significantly associated with the disease. The strong association was further evident by selecting one of these markers, rs965513, with the genotyping of an independent set of samples by Taqman (overall mhp = 4.8×10^{-12}, OR = 1.65, 95% CI: 1.43–1.91). Rs965513 was recently identified as a genetic risk factor for sporadic PTC in individuals of European descent (11) and is located within an LD block which lies centromeric to *FOXE1*.

Another recent report showed a strong association of rs1867277 at the 5′-UTR of *FOXE1* with the risk of differentiated thyroid cancer, in particular with the classic variant of PTC. *FOXE1* is a thyroid-specific DNA binding protein recognizing binding sites on thyroglobulin and thyroperoxidase genes expressed in thyroid follicular cells (14,15). Although the precise role of *FOXE1* in PTC remains to be fully established, this study provides further evidence of *FOXE1* involvement in thyroid carcinogenesis. Rs1867277 is so far the only functional variant associated with sporadic PTC identified within the *FOXE1* gene, and the risk allele (A) augmented *FOXE1* transcription by creation of a binding site for USF1 and USF2 transcription factors. The fact that stronger association signals were observed for SNPs outside block C containing *FOXE1* in both the Icelandic and Belarusian studies may indicate the existence of DNA sequences in block B with unknown function acting cooperatively with rs1867277. Certainly, however, we cannot rule out the involvement of other genes in the region.

Although the association of rs965513 with PTC was stronger in the early-onset cases than in the late-onset cases, the difference was not statistically significant (p-heterogeneity = 0.063). Short latency was reported to be often associated with more aggressive tumors with prominent local invasion and distant metastases (16). However, it is difficult to directly associate our results to such morphological features since the environmental background of patients, including individual thyroid radiation dose and detailed clinical information are not available.

Individual susceptibility to thyroid cancer is considered to be complex involving the interaction of low-penetrance genes and the environment. Here we provide the first evidence

Table 2. Results of association analysis for SNP markers at 9q22.33 and 14q13.3 using the Chernobyl childhood thyroid cancer cohort

Marker	Allele[a]		Chr	Position	Statistics by study					Study 1 + 2		Study 1 + 2 + 3		Gudmundsson *et al.*			
	Ref.	Var			Study	Freq var. Case	Freq var. Control	Trend P[b]	OR (95% CI)[c]	Mhp[d]	OR (95% CI)[c]	Mhp[d]	OR (95% CI)[c]	Freq var. Case	Freq var. Control	P-value[e]	OR (95% CI)[c]
rs1512261	G*	T	9	99562351	1	0.500	0.419	0.029	1.39 (1.03, 1.87)	6.9×10^{-6}	1.53 (1.27, 1.84)						
					2	0.509	0.391	5.2×10^{-5}	1.62 (1.28, 2.04)								
rs1877432	G*	A	9	99583701	1	0.706	0.610	0.010	1.53 (1.12, 2.09)	5.2×10^{-4}	1.40 (1.16, 1.69)						
					2	0.697	0.628	0.016	1.36 (1.06, 1.74)								
rs925489	C*	T	9	99586421	1	0.487	0.334	3.3×10^{-5}	1.89 (1.40, 2.55)	1.7×10^{-9}	1.79 (1.48, 2.16)						
					2	0.481	0.349	6.0×10^{-6}	1.73 (1.37, 2.18)								
rs7850258	A*	G	9	99588834	1	0.487	0.334	3.3×10^{-5}	1.89 (1.40, 2.55)	1.7×10^{-9}	1.79 (1.48, 2.16)						
					2	0.481	0.349	6.0×10^{-6}	1.73 (1.37, 2.18)								
rs965513	A*	G	9	99595930	1	0.487	0.334	3.3×10^{-5}	1.89 (1.40, 2.55)	4.9×10^{-9}	1.76 (1.45, 2.12)	4.8×10^{-12}	1.65 (1.43, 1.91)	0.490	0.352	6.8×10^{-20}	1.77 (1.57, 2.00)
					2	0.476	0.352	1.7×10^{-5}	1.68 (1.33, 2.12)								
					3	0.462	0.367	2.0×10^{-4}	1.48 (1.20,1.83)								
rs10759944	A*	G	9	99596793	1	0.487	0.334	3.3×10^{-5}	1.89 (1.40, 2.55)	3.5×10^{-9}	1.77 (1.46, 2.14)			0.490	0.352	1.7×10^{-19}	1.77 (1.57, 2.01)
					2	0.479	0.352	1.2×10^{-5}	1.69 (1.34, 2.14)								
rs7848973	A*	G	9	99628660	1	0.503	0.392	0.0032	1.56 (1.16, 2.10)	2.5×10^{-6}	1.56 (1.29, 1.87)						
					2	0.502	0.393	2.0×10^{-4}	1.56 (1.24, 1.97)								
rs7024345	A*	G	9	99635059	1	0.380	0.305	0.038	1.39 (1.02, 1.90)	1.4×10^{-6}	1.63 (1.33, 1.98)			0.387	0.285	1.9×10^{-12}	1.58 (1.39, 1.80)
					2	0.397	0.273	4.0×10^{-6}	1.75 (1.37, 2.23)								
rs1443434	G*	T	9	99657300	1	0.487	0.392	0.012	1.47 (1.09, 1.97)	2.6×10^{-4}	1.41 (1.17, 1.70)			0.488	0.385	2.8×10^{-9}	1.52 (1.32, 1.74)
					2	0.477	0.398	0.0070	1.37 (1.09, 1.73)								
rs907580	T*	C	9	99662418	1	0.374	0.314	0.10	1.30 (0.96, 1.78)	5.7×10^{-6}	1.58 (1.30, 1.92)			0.395	0.281	1.1×10^{-14}	1.66 (1.46, 1.89)
					2	0.396	0.273	4.2×10^{-6}	1.74 (1.37, 2.23)								
rs925487	C*	T	9	99676219	1	0.463	0.372	0.015	1.45 (1.08, 1.96)	4.5×10^{-5}	1.47 (1.22, 1.77)			0.472	0.359	2.6×10^{-13}	1.60 (1.41, 1.81)
					2	0.465	0.369	9.2×10^{-4}	1.48 (1.17, 1.88)								
rs10984103	A*	C	9	99679096	1	0.463	0.372	0.015	1.45 (1.08, 1.96)	4.6×10^{-5}	1.47 (1.22, 1.77)			0.472	0.359	2.2×10^{-13}	1.59 (1.41, 1.81)
					2	0.465	0.369	9.5×10^{-4}	1.48 (1.17, 1.87)								
rs7866436	G*	A	9	99689917	1	0.465	0.369	0.010	1.49 (1.10, 2.00)	5.2×10^{-5}	1.47 (1.22, 1.76)						
					2	0.463	0.372	0.0016	1.46 (1.15, 1.84)								
rs944289	C	T*	14	35718997	1	0.626	0.580	0.23	1.21 (0.90, 1.63)	0.17	1.13 (0.95, 1.36)			0.644	0.558	2.5×10^{-8}	1.44 (1.26, 1.63)
					2	0.607	0.584	0.43	1.10 (0.87, 1.40)								

SNP markers in blocks B and C with $P < 1 \times 10^{-3}$ are shown for 9q22.23. Rs944289 on 14q13.3 which also showed significant association in the Icelandic study was included. A complete list of the markers in the 425-kb region with statistical results is shown in Supplementary Material, Table S1.

[a]The reference (ref.) and variant (var.) alleles refer to NCBI Build 36.3 and the risk allele is indicated with an asterisk.

[b]The P-values using Trend χ^2 test are shown.

[c]Odds ratio (OR) is calculated for the risk allele with a confidence interval (CI) of 95%.

[d]The Trend χ^2 Mantel–Haenszel P-values are shown.

[e]The P-values using a standard likelihood ratio χ^2 statistic are shown.

that the risk of developing PTC after internal radiation exposure is largely associated with the genetic determinant conferring risk for human thyroid malignancies in the general population. However, *FOXE1* is unlikely to be the only key player in radiation-related thyroid carcinogenesis and it remains to be established whether or not radiation-related PTC has other etiology-specific genetic components for inherited predisposition. Rs944289 at chromosome 14q13.3 strongly associated with sporadic PTC in the Icelandic population was not significant in our results. Moreover, in our GWA study, two additional SNPs with meta-analysis *P*-value being smaller than 1×10^{-6} were identified, of which one was on chromosome 9p and the other on chromosome 12p. Since neither of these chromosomal loci have been identified as being associated with sporadic PTC, they may be potential candidates for susceptibility loci specific to radiation-related PTC. These observations clearly suggest that different genetic components are involved in carcinogenesis of sporadic and radiation-related PTC.

Only a few case–control studies to identify genetic risk factors of radiation-related thyroid cancer have been reported to date. Three studies included Chernobyl PTC (17–19) and thyroid cancers in an occupationally exposed cohort (20). A recent article examined the genetic determinants in the patients with radiation-related thyroid nodules (21). The possibilities of association between the risk for PTC after radiation exposure and *TP53* (ID: 7157) (17,18), *RET* (ID: 5979) (20) or *XRCC1* (ID: 7515) (20) were demonstrated. However, most of these studies had a limited sample size and insufficient gene coverage. Apart from the *TP53* Arg72Pro polymorphism (rs1042522) being associated with the risk of radiation-related PTC in adult patients (17,18), the findings were not replicated in independent sample sets. None of the SNP markers that were significant in the above studies were on the Illumina array. According to HAPMAP, rs25487 (*XRCC1*) and rs1800858 (*RET*) are in complete LD ($D' = 1$, $r^2 = 1$) with rs1799778 and rs2505535, respectively, which are both on the array. However, the associations were negative for both markers in our study ($P = 0.94$ for rs1799778 and $P = 0.03$ for rs2505535).

MATERIALS AND METHODS

Study populations

A total of 667 patients (174 males and 493 females, sex ratio 0.35) diagnosed for thyroid cancer in 1989–2009 were recruited. Inclusion criteria for cases were as follows: (i) age at the time of Chernobyl accident 0–18 years old, including those *in utero*, in April–June 1986, who were (ii) residing at the time in the radiocontaminated regions of Belarus and (iii) histologically verified diagnosis of PTC. Demographic and diagnostic information was retrieved from Thyroid Cancer Center (Minsk, Belarus). At the moment of exposure, 378 patients were residents of Gomel region of Belarus which is the most radiocontaminated area in the country, 195 patients were from Brest region, 10 from Mogilev region and 84 were from other radiocontaminated regions of the country.

As control subjects, a total number of 620 healthy individuals (165 males and 455 females, sex ratio 0.36) were recruited. Inclusion criteria for controls were: (i) age at the time of accident between 0 and 18 years old, including those *in utero*, in April–June 1986, who were (ii) residing at the time in the radiocontaminated regions of Belarus, (iii) euthyroid state and (iv) no thyroid cancer by the time of sampling (February 2006 to April 2009). At the time of possible radiation exposure, 574 healthy participants were residents of Brest region, 34 of Gomel region, 11 of Mogilev region and one individual from another region. According to the radioecological and radiation epidemiology studies, all cases and 620 controls are considered to have received thyroid doses ranging 21–1500 mGy (22,23). Additional DNA samples of 207 individuals who were: (i) born after 1987 (79 samples), (ii) older than 18 years of age at the time of accident (three samples) or (iii) considered to have been exposed to a negligible amount, if any, of radiation according to their residential information (125 samples), were also utilized for the studies as representative Belarusian population controls. Demographic and residential information was obtained by personal inquiry, and peripheral blood samples were collected in the contaminated regions during bi-annual thyroid screening programs (which also included neck ultrasound and consultation of endocrinologist) of Belarusian population. Euthyroid state was confirmed by laboratory tests being 1.64 ± 1.57 μU/ml for thyrotropin (normal range 0.5–5.0 μU/ml) and 1.17 ± 0.28 ng/dl for free thyroxin (normal range 0.7–1.55 ng/dl) in the whole control group. The absence of thyroid cancer was met by selecting only those individuals without detectable thyroid nodules on ultrasound. For Study 2, the genotypes of 448 Russian controls were used as population controls (24). The Institutional Review Board and the Ethics Committee of each institution approved the protocols used. All participants were fully informed of the purpose and procedures, and a written consent was obtained.

DNA preparation

DNA was extracted from peripheral blood mononuclear cells using Puregene kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. DNA concentration and purity were measured with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The samples were stored at −80°C until use.

GWA study

Two series of genome scans were performed using two independent sample sets. 194 cases and 179 controls, and 214 cases and 448 Russian population controls, were used in the first and second genome scans (Study 1 and Study 2), respectively. Validation of genome scan results (Study 3) was performed by Taqman analysis using a third independent sample set consisting of 259 cases and 648 controls.

Study 1: genome scan. A total of 567 512 autosomal SNPs were genotyped in 194 thyroid cancer patients and 179 controls with Illumina Human610-Quad BeadChip on a BeadStation 500G Genotyping System, and genotype calls were generated and summary files were made using the Bead Studio version 3.1.3.0 software package (Illumina, Inc., San

Diego, CA, USA). Quality control procedures were systematically performed for the genome scan results. Initially, two control samples with call rates being smaller than 90% were removed from the analysis. Subsequently, degrees of kinship between individuals were examined by Pi-hat in PLINK, a multidimensional scaling method (25). For seven pairs of cases and five pairs of controls showing high degrees of kinship (PI-HAT > 0.3), the sample with the lower call rate was excluded. Principal component analysis by 'smartpca' in EIGENSOFT (26) including HAPMAP phase II samples confirmed no deviation in all DNA samples from Caucasian population. Following the quality control for SNP markers, a total of 35 488 markers were excluded due to low call rates (lower than 95%), a low minor allele frequency (smaller than 0.01) or significant distortion from Hardy–Weinberg equilibrium (*P*-value smaller than 10^{-7}). After these steps, 532 024 SNP markers of 187 PTC patients (mean age $\pm$ SD: 3.0 ± 3.8 years) and 172 controls (1.5 ± 2.8 years) were used for statistical analyses.

Association of SNP markers on the X chromosome was examined in a separate analysis. The same criteria for QC were applied and 16 448 SNP markers were used to test disease association between 58 cases and 60 controls for males, and 128 cases and 111 controls for females.

Study 2: genome scan. In 214 thyroid cancer patients (mean age $\pm$ SD: 5.8 ± 5.2 years), 567 512 autosomal SNPs were genotyped using the same SNP arrays as those used in Study 1. Genotype calls of 448 Russian DNA samples were used as population-based controls. The same exclusion criteria as Study 1 were applied for the quality control, but no DNA samples were removed from the analysis. After removing 57 902 SNP markers that fit the exclusion criteria, a total of 509 610 SNP markers were used for statistical analyses. Analysis of the X chromosome was performed as described for Study 1, in 52 cases and 235 controls for males and in 161 cases and 213 controls for females.

Study 3: validation analysis. Validation of genome scan results was carried out in 259 cases (mean age $\pm$ SD: 6.8 ± 5.5 years) and 648 controls (mean age $\pm$ SD: 6.2 ± 5.9 years) using the Taqman SNP assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines. A predesigned and functionally tested probe was used for rs965513 (C_1593670_20, Applied Biosystems), and a custom designed probe by the same producer was used for rs1867277.

Statistical analysis

A case–control association in each study was examined using trend χ^2 test to compare genotypic distributions between cases and controls (27). Population stratification was assessed by the genomic control method (28). Meta-analysis of genome scan results was carried out with trend mode of the Mantel–Haenszel method (29), by combining the genotypes of Study 1 and Study 2 for 506 840 autosomal SNP markers that passed quality control in both studies. The genotypes for the autosomal SNPs obtained in Studies 1 and 2 were pooled, and population stratification was corrected by Eigenstrat (26) followed by the genomic control method. Meta-analysis of 16 448 SNP markers on the X chromosome was performed for Study 1 males and Study 2 males, as well as for Study 1 females and Study 2 females.

The overall significance level of rs965513 was calculated by meta-analysis using the Mantel–Haenszel method, combining the genotypes of Study 1 to Study 3. The LD structure was derived using the genotypes of Study 1 and Study 2 using the Haploview software (30) by calculating pairwise LD indices (D' and r^2) between SNP markers in the region.

Imputation of missing genotypes was performed using MACH 1.0 (http://www.sph.umich.edu/csg/abecasis/MaCH/index.html). The genotype data of CEU (CEPH European) obtained from the Phase III HapMap database (draft2) were used as reference and the 425-kb region between rs4742698 and rs4618817 was examined for association. In the process of imputation, 50 Markov chain iterations were implemented.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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