Introduction

Ionizing radiation is a well known genotoxic agent that induces a variety of DNA lesions including nucleotide base modifications, abasic sites, strand cross-linking, DNA adducts, and single- and double-strand DNA breaks (DSBs) (1-3). Although all these types of lesions may potentially result in gene mutations, DSBs are considered to be the most significant for chromosomal aberrations, mutagenesis, genetic instability and carcinogenesis (2, 4-7). The multiplicity of DNA damages produced by radiation is thought to be one of the reasons for the diversity in biological consequences of exposure.

Human thyroid is an organ particularly vulnerable to ionizing radiation as was initially seen in the series of patients subjected to external beam therapy of the head and neck area for medical indications who then developed thyroid cancer (8). The Chernobyl accident, which occurred nearly 25 years ago on April 26, 1986, provided evidence of carcinogenic effect of environmental exposure to radioiodine isotopes, especially to ^{131}I. A significant increase in thyroid cancer incidence was documented since early 1990-ies in Belarus, Ukraine and southwestern regions of Russia (9-12) (Fig. 1).

By 2002, the number of thyroid cancer cases registered in the individuals aged less than 18 years at the moment of exposure in the three most affected countries approached to 5,000 (13). Epidemiological studies have established qualitative and quantitative characteristics of causative association of thyroid cancer risk with internal exposure to radioiodine demonstrating that it is comparable to that after external irradiation (11, 14-20).

The outbreak of thyroid cancer in young patients suffered from the radioactive Chernobyl fallouts led to a great number of medical, epidemiological, dosimetric, sociological and laboratory investigations all aimed at evaluation of health impact, short and long-term consequences of the catastrophe for individuals, society and the environment as well as at elucidating the distinctive features of radiation-induced tumors. They resulted in important evidence-based conclusions which may be called lessons from Chernobyl; some of them could be drawn only after decades of observations. Applicably to the thyroid, the most important would be that ingestion of ^{131}I at childhood may later cause thyroid cancer, that period of latency after exposure may be as short as only 4 years, that the use of stable iodine as a dietary supplement or as a thyroid-blocking agent may have a protective effect against cancer. From the molecular and pathological point of view, it has been recognized that radiation excess in thyroid cancer incidence is due to the papillary thyroid carcinoma (PTC) whose morphology and molecular characteristics, such as histological architecture and mutational pattern, appear to be changing with increasing latency or correlate with patient's age (see reff. 20, 22, 23 for extensive reviews). The relative prevalence of *RET/PTC3*, *RET/PTC1* and *BRAF* mutations implicated in molecular carcinogenesis of PTC has been proposed to tentatively parallel the dynamics of thyroid cancer incidence in children, adolescents and adults, respectively, shown in Fig. 1 (20).

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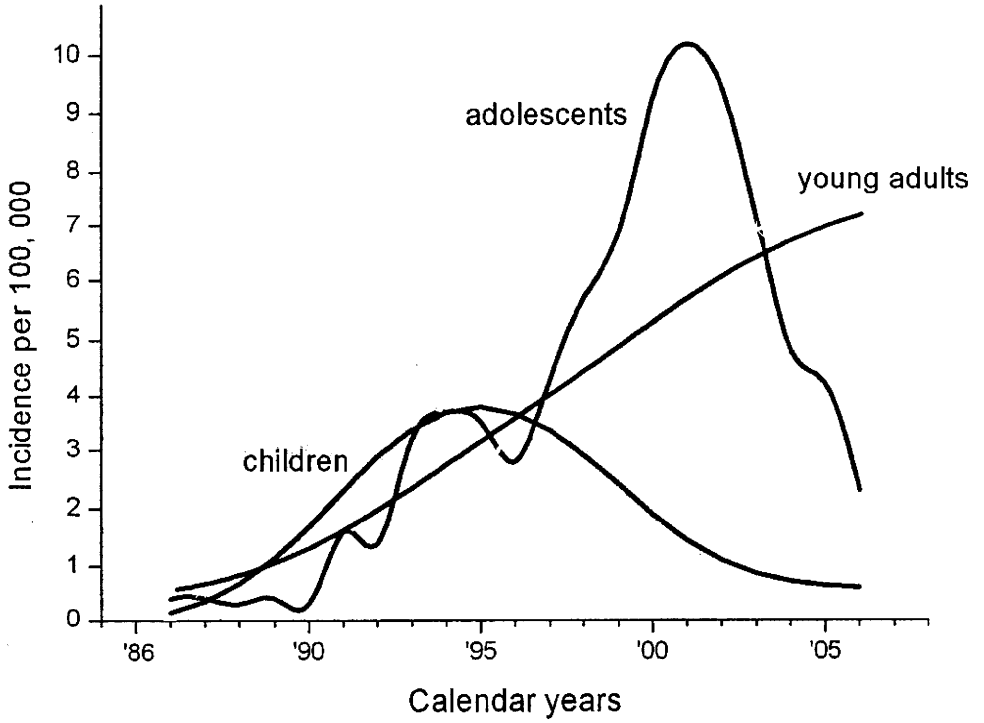

Fig. 1. Incidence of thyroid cancer in Belarus among the residents of radiocontaminated territories by age groups. This graph is inferred from the original one published earlier (21).

Molecular studies in Chernobyl thyroid cancer, depending on design, could be broadly classified into those attempting to determine a "damage signature" or "susceptibility signature" (24-26). The first type of investigations explores frequencies and distribution of various mutations, in a comparative manner, between radiation-induced and sporadic thyroid cancers. Initial works on Chernobyl series were mostly mutational studies. As a whole, they demonstrated that none of oncogenes such as gene rearrangements (*RET/PTC*, *NTRK*, *AKAP9-BRAF*) or point mutations (*BRAF*, *RAS* family genes) could have been identified as radiation-specific.

Studies of the second type investigate if gene expression patterns or genetic factors may modify or serve as markers of inherited predisposition for developing cancer after radiation exposure. They generally require more advanced techniques because of the need to cover a large number of targets, ideally the whole genome. So far, several factors have been established to affect risk for developing thyroid cancer following internal exposure: radiation dose for the thyroid, younger age at exposure and iodine deficiency. Whether or not the genetics particularities of the individuals who developed thyroid cancer after Chernobyl remains largely unknown, but some facts, such as inter-

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patient variations in the clinical course and latency as well as development of cancer only in a small proportion of the exposed victims, may be indicative of such a possibility.

In this review we focus on the works performed to establish molecular classifiers capable of distinguishing radiation-induced Chernobyl cancers form sporadic PTCs. The importance and a need of a classifier is determined by the necessity to improve radiation risk assessment and risk communication, as well as to better manage and justify occupational and medical exposures tending to be expanding in the modern era of nuclear technologies.

Chromosomal imbalances

In an early study, chromosomal imbalances were examined using conventional comparative genomic hybridization (CGH) in a group of 60 Chernobyl childhood and adolescent PTCs (27). About 30% tumors were found to carry copy number variation (CNV). Both DNA gains (chromosomes 2, 7q11.2-21, 13q21-22, 21) and losses (16p/q, 20q, 22q) were found. Interestingly, deletions or loss of heterozygosity (LOH) on chromosomes 22q and 16p/q have been reported previously in PTC, FTC or ATC and associated with an aggressive tumor behavior (28-30). This study did not reveal correlations between the *RET/PTC* status of a tumor and specific DNA imbalance, yet the observation of a deletion at 22q in both *RET/PTC*-positive and *RET/PTC*-negative tumors was suggestive of the existence of alternate routes contributing to carcinogenesis, genetic heterogeneity or oligoclonal tumor development. The latter suggestion is supported by the observation of non-homogenous distribution of *RET/PTC*-harboring nuclei across tumor tissues (31). In a later work of the same group, employing an BAC-based array CGH, it was shown that *RET/PTC*-positive and *RET/PTC*-negative cases could be discriminated by the alteration pattern of chromosomes 1p, 3q, 4p, 7p, 9p/q, 10q, 12q, 13q and 21q (32). Furthermore, there was a significant difference between *RET/PTC*-positive childhood and adult PTCs: deletions on 1p35–36 were more frequent in adult cases. Regardless of *RET/PTC* rearrangement, chromosomal losses were more common than gains. In line with the previous study, the existence of additional, sometimes multiple, DNA alterations in both *RET/PTC*-positive and in *RET/PTC*-negative tumors could be interpreted as pointing at alternative paths of tumor development.

Another CGH study of 23 Chernobyl and 20 sporadic PTCs demonstrated that the overall prevalence of DNA gains was 2-4 higher in exposed patients as compared to non-exposed, and even more frequent (up to 10-fold) for recurrent gains (33). It was possible to determine the alteration pattern that discriminated radiation-related PTCs from sporadic (chromosomes 1p36.32-.33, 2p23.2-.3, 3p21.1-.31, 6p22.1-.2, 7q36.1, 8q24.3, 9q34.11, 9q34.3, 11p15.5, 11q13.2-12.3, 14q32.33, 16p13.3, 16p11.2, 16q21-q12.2, 17q25.1,19p13.31-qter, 22q11.21, 22q13.2) but because of limited sample size and non-uniform distribution of individual thyroid doses in the investigation the assessment of dose-response relationship has proved difficult. It was concluded that CNV, in addition

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to carcinogenesis-related alterations, also depend on radiation exposure and patient's age at exposure.

Using a 50K Mapping array, 10 childhood Chernobyl PTCs were recently analyzed to demonstrate that DNA gains were more consistently observed at chromosome 1p, 5p, 9q, 12q, 13q, 16p, 21q, and 22q, while losses were found at 1q, 6q, 9q, 10q, 13q, 14q, 21q, and 22q (34). CNV amplifications were more frequent than deletions in line with the study by Kimmel et al. (33); no significant LOH was registered. This study is interesting because an overlay analysis was done to evaluate the concordance between CNV and gene expression. As a result, none of genes mapped to deleted regions was found to be downregulated. On the contrary, 87 genes that were amplified on CGH also displayed overexpression. After filtering gene expression profiles in Chernobyl PTCs against those reported previously for sporadic tumors and available from Gene Expression Omnibus, a radiation-related PTC identifier was established that included 113 messages among which 24 were downregulated and 41 were upregulated at least 3-fold. Six genes, *CAMK2N1*, *AK1*, *DHRS3*, *FBXO2*, *ECE1* and *PDE9A* were unique to childhood radiation-induced PTC.

As a whole, the results of CGH analyses performed to date are not yet comprehensive enough to derive a CNV-based radiation signature. Usually the studies deal with small sample size, do not report validation experiments on independent specimens and employ platforms that are quite different in their resolution cumulatively making cross-analysis difficult. They, however, provide insights into the genomic regions, candidate genes and functional pathways involved in radiation-related thyroid carcinogenesis.

Gene expression profiles

Several studies have been undertaken to elucidate characteristic expressiosome features of Chernobyl thyroid cancers. The earliest one analyzed 12 Ukrainian and 8 sporadic PTCs from French patients, and 13 thyroid adenomas using Micromax microarrays with a set of 2400 known human cDNA probes (24). Neither unsupervised nor supervised classification algorithms could distinguish radiation-related from sporadic PTCs, perhaps in part due to the relatively small number of tested genes. However, separation from benign thyroid neoplasia was effective: based on a 36-gene signature a 3% misclassification rate was achieved. The importance of this investigation was in obtaining molecular evidence of similarity between PTCs of different etiology which confirmed previous observations of their morphological resemblance once again proving that radiation-induced and sporadic PTCs are closely related diseases presumably having much in common pathogenetically.

The whole genome study used Human Genome Survey Microarray V2.0 platform that combines >29000 genes (35). Screening was done on pooled RNA samples from 11 Chernobyl patients aged 15-22 years at diagnosis and 41 patients from southeastern Germany aged 15-83 years and the results were confirmed on an RTQ-PCR low-density array for selected genes.

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Microarray analysis detected 646 differentially upregulated and 677 downregulated genes (>5-fold difference) between the groups. Interestingly, the genes predominantly overexpressed in Chernobyl tumors included G-proteins (RAS family genes), growth factors and receptors (*VEGFA*, *EGFL9*, *PDGFC*, *PDGFRB*, *IGF1R*, *IGBP1*) and some of oxidoreductases (cyclooxygenase 2 (*PTGS2*), superoxide dismutase (*SOD1*)) which were associated with tumor aggressiveness and poorer prognosis in previous studies (36-42). Such overexpression was interpreted as supportive to the notion that Chernobyl PTC manifested particularly high aggressiveness with frequent lymph node metastases and extrathyroidal invasion. This work also identified a molecular classifier consisting of 7 genes (*SFRP1, MMP1, ESM1, KRTAP2-1, COL13A1, BAALC* and *PAGE1*) that enabled a confident classification into radiation-related and sporadic PTCs.

One more investigation explored transcriptomes in 12 Chernobyl and 14 French patients using Human 1 cDNA Microarray slides covering 8000 genes (43). Similarly to the previous report from this group (24), unsupervised classification did not provide distinction between the two groups of cancers on a global scale. A supervised analysis, however, using four different algorithms, succeeded to determine classifiers that included from one to several thousands genes (median 256) with overall error rates ranging 12-27%. This study is noteworthy because the effects of possible etiological agents, which are presumably gamma radiation in Chernobyl tumors and hydrogen peroxide in sporadic tumors, were taken into account. Hydrogen peroxide is produced during thyroid hormone synthesis (44) and may play a role in thyroid tumorigenesis (45). Furthermore, it is a potent DNA-damaging substance which produces not only single-strand DNA breaks and base modifications but also double-strand breaks and, as recently shown, is capable to generate *RET/PTC1* rearrangement in a human thyroid cell line (46). Using previously available data (47), the authors found that in a B-lymphocyte cell line treated with 10 different genotoxic agents, *in vitro* gene expression responses to 200 μM of hydrogen peroxide and 2.5 Gy of gamma-rays were the most resembling. There were however 293 genes whose expression levels differed >1.5-fold between the two types of treatment of which, after removing genes related to immune reactions, 118 were present on the arrays used to profile PTCs. These genes were tested as a molecular classifier and, as a result, led to the separation of Chernobyl and sporadic PTC with the error rates 15-27%. In addition, whether the genes whose products are involved in five major DNA repair mechanisms, i.e. base-excision repair, mismatch-excision repair, nucleotide-excision repair, homologous recombination and nonhomologous end joining, may constitute a classifier was explored. Thirteen genes of homologous recombination pathway were found to make a classifier that distinguished radiation-induced and sporadic PTCs with error rates of 15-31%. It was proposed that, given DNA repair is largely accomplished within hours after damage while differential gene expression in the tumors persisted for many years, such profile may be a signature of susceptibility to different etiological forms of thyroid cancer. If these results find further support in independent PTC series, they may well be considered as a piece of evidence suggesting the existence of inherited predisposition to radiation-induced PTC.

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Similarly to the results obtained in CGH studies, gene expression data provide valuable information for the attempts of elucidating molecular radiation signature, but they are not completed yet. So far reported works, being generally encouraging, have been done using relatively small series of cancers and produce the results that do not converge to yield a reliable set of markers. This points at the need to expand the number of analyzed cancers of both etiologies with better matching in terms of clinico-pathological and molecular characteristics to achieve the desired reproducibility and avoid biases.

Proteomic investigation

To date only one proteomic study involving Chernobyl thyroid cancers has been reported to the best of our knowledge. Boltze et al. analyzed protein extracts from 86 Chernobyl and 91 sporadic PTCs from patients of southeastern Germany (48). On 2-D electrophoresis, around 2000 spots were identified on the reference gels and among them 18 candidates upregulated in radiation-induced PTCs were determined. Immunohistochemistry was performed for all these candidates and in addition for two other proteins, potential markers for PTC. The results were evaluated semiquantitatively eventually leaving 6 proteins (NTRK1, MMP-1, MMP-13, MMP-9, Cathepsin W and Cathepsin X) that allowed most efficient separation between the groups. When adjusted for patients' age, NTRK1, MMP-1 and MMP-13 staining resulted in a complete separation of the two etiological groups. Without age adjustment, NRTK1 alone and a combination of either two MMPs or of two Cathepsins also worked well with no false positive and false negative test results. Note that *MMP1* gene upregulation in Chernobyl PTCs was reported previously (35). Interestingly, NTRK1 overexpression in radiation-induced PTCs may indicate structural mutation-independent role of this receptor tyrosine kinase as chromosomal rearrangements involving the *NTRK1* gene are observed in less than 10% of Chernobyl cancers (49).

Whether a relatively simple immunostaining approach can be universally used to discriminate radiation-induced from sporadic PTCs remains to be established. Concerns are related first of all to patients' age (and/or duration of latent period) and associated changes in tumor morphology as well as underlying mutational events all potentially leading to the shifts in the spectrum of expressed proteins. This direction certainly needs further investigation.

Genetic association studies

The purpose of this type of investigations is to determine genetic factors associated with disease thus addressing issue of inherited susceptibility. In general, there are two methodologies of selecting gene polymorphisms, usually SNPs, to be analyzed. The first one, termed candidate gene approach, is based on a hypothesis that genetic variations in one or in a limited number of genes may affect risk for or the phenotype of a given disease. A more comprehensive way is initially hypothesis-free and employs analysis throughout the genome; it is termed genome-wide association study

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(GWAS). While a substantial number of studies has been done in sporadic thyroid cancers, only few explored radiation-induced thyroid malignancies.

Candidate gene approach

In a study by Stephens et al. (50) no evidence for LOH in the *RET* gene was found in 28 of 46 PTCs from Ukraine heterozygous for at least one of three SNPs of interest (G691S, S904S and L769L); this observation is in line with the later microarray findings (33). Investigation of the additional 68 cases demonstrated that the rare S allele of G691S was significantly overrepresented in patients aged more than 30 years (30-72 years old, range and exposed 10-14 years before operation) as compared to the younger ones. Since excess radiation risks for PTC in the individuals exposed at the age older than 20 years old is very low and further declines with age at exposure, it was proposed that *RET* polymorphisms may influence carcinogenesis in sporadic but not in radiation-induced PTCs.

The Arg72Pro polymorphism of the *TP53* gene (encodes tumor suppressor protein p53) was assessed in 48 pediatric/adolescent and 68 adult Ukrainian and Russian patients with PTC, residents of radiocontaminated territories in Chernobyl areas (51), and 53 adult patients with sporadic PTC and 313 healthy controls from Russia. The Arg/Arg homozyotes were found to be significantly underrepresented in adult patients, but not in children and adolescents. In tumor tissues, no LOH or imbalanced *TP53* allele expression in heterozygous individuals was found. These findings suggested that germline *TP53* allele combinations other than Arg/Arg may contribute to the risk of development of PTC in individuals exposed to radiation during their late childhood, adolescence or in young adulthood, particularly females aged between 18 and 30. Of note, elevated risk for thyroid cancer was reported in females exposed to Chernobyl radiation at the age below 30 years in an epidemiological investigation (52).

A recent study of 9 SNPs in 5 genes (*ATM*, *XRCC1*, *TP53*, *XRCC3* and *MTF1*) involved in DNA damage response in 255 PTC patients (123 from Chernobyl areas and 132 sporadic) and 596 healthy controls (198 residents of Chernobyl areas and 398 subjects without history of radiation exposure) showed that the *ATM* G5557A and *XRCC1* Arg399Gln polymorphisms, regardless of radiation exposure, were associated with a decreased risk of cancer (53). Interestingly, the *ATM* IVS22-77 T>C and *TP53* Arg72Pro SNPs interacted with radiation exposure: the *ATM* IVS22-77 associated with the increased risk of sporadic PTC whereas *TP53* Arg72Pro correlated with the higher risk of radiation-induced PTC in adult patients, in support to the previous report (51). A possibility of gene-gene and gene-environment interactions was demonstrated. Some particular *ATM*/*TP53* genotypes strongly associated with either sporadic or radiation-induced cancer indicating that variability of these genes may be potential risk modifiers for developing PTC of different etiology.

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Molecular epidemiology based on whole genome association data

To date only one investigation of Chernobyl PTCs employing GWAS has been published (54). A total of 667 patients from Belarus diagnosed for PTC in 1989–2009 and 1275 controls from Belarus and Russia were studied, of which 408 cases and 627 controls were genotyped using Illumina Human610-Quad BeadChips (>500,000 SNPs) and the remaining samples were used for validation study. Statistical meta-analysis identified 4 SNPs at chromosome 9q22.33 showing significant association with disease. For one of them, rs965513, used for validation, a *P*-value of 4.8×10^{-12} was obtained which far surpasses the threshold of genome-wide significance of 5×10^{-8} (55). This SNP is located within a linkage disequilibrium (LD) block centromeric to the *FOXE1* gene which encodes a thyroid-specific transcription factor TTF2 playing pivotal roles in thyroid morphogenesis. In addition, two candidates SNPs on chromosomes 9p and 12p that strongly tended to associate with disease risk were identified but genotyping of additional samples would be necessary to validate the significance of those.

To better understand the importance of this finding, it is necessary to mention two studies of genetic predisposition to sporadic differentiated thyroid cancer published last year just before the study by Takahashi et al. The first one reported rs965513, the same polymorphism described in the Chernobyl series, as the strongest genetic marker associating with thyroid malignancy in individuals of European descent. This study also claimed another SNP, rs944289 on chromosome 14q13.3 in the proximity of the *NKX2-1* gene that encodes the TTF1 transcription factor, to be a marker for thyroid cancer (56) but it was not confirmed in the Chernobyl series. The second study, employing candidate gene approach, initially genotyped 768 SNPs in 97 genes in 615 cases and 525 controls from Spain and used 482 patients and 532 controls from Italy for validation (57). The target genes were selected based on their differential expression in primary thyroid tumours or the involvement in thyrocyte biology, metabolism and/or carcinogenesis such as the MAP kinase, JAK/STAT and TGF-beta pathways. An SNP, rs1867277, within the LD block spanning *FOXE1* and located at the 5'UTR of the gene was identified as associating with PTC. Functional study demonstrated that this SNP affects *FOXE1* expression by recruiting the USF1/USF2 transcription factors. Since forkhead transcription factors have been implicated in several human cancers (58-61) including epithelial-mesenchymal transition in colon cancer (62), it was proposed that FOXE1 may influence thyroid tumor call migration and invasion. While its precise role remains to be elucidated, this was an important clue to the understanding the molecular pathogenesis of PTC.

Thus, the three studies, two of sporadic thyroid cancers and one of radiation-induced tumors, have concordantly identified the *FOXE1* (*TTF2*) locus as a marker of inherited susceptibility for PTC of different etiology. This leads to an important corollary that among the genetic factors affecting risk for radiation-induced Chernobyl PTC the strongest one is the same that confers predisposition to the sporadic form of this type of malignancy. Therefore, it is likely that "radiation-sensitive genotype", whose existence may be expected given the possible existence of putative radiation-associated

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markers on chromosomes 9p and 12p and the absence of sporadic PTC marker on 14q13.3 (i.e. *NKX2-1* or *TTF1*), comes next to and after, in terms of the effect strength, the general susceptibility to thyroid cancer. As outlined in Fig. 2, the results of genetic association studies allow to add genetic predisposition to the list of risk factors for radiation-induced thyroid carcinogenesis known from the earlier experience. Further investigation of etiology-specific marker(s) will probably refine our understanding of radiation-induced carcinogenesis by addressing issues of gene-gene and gene-environment interactions.

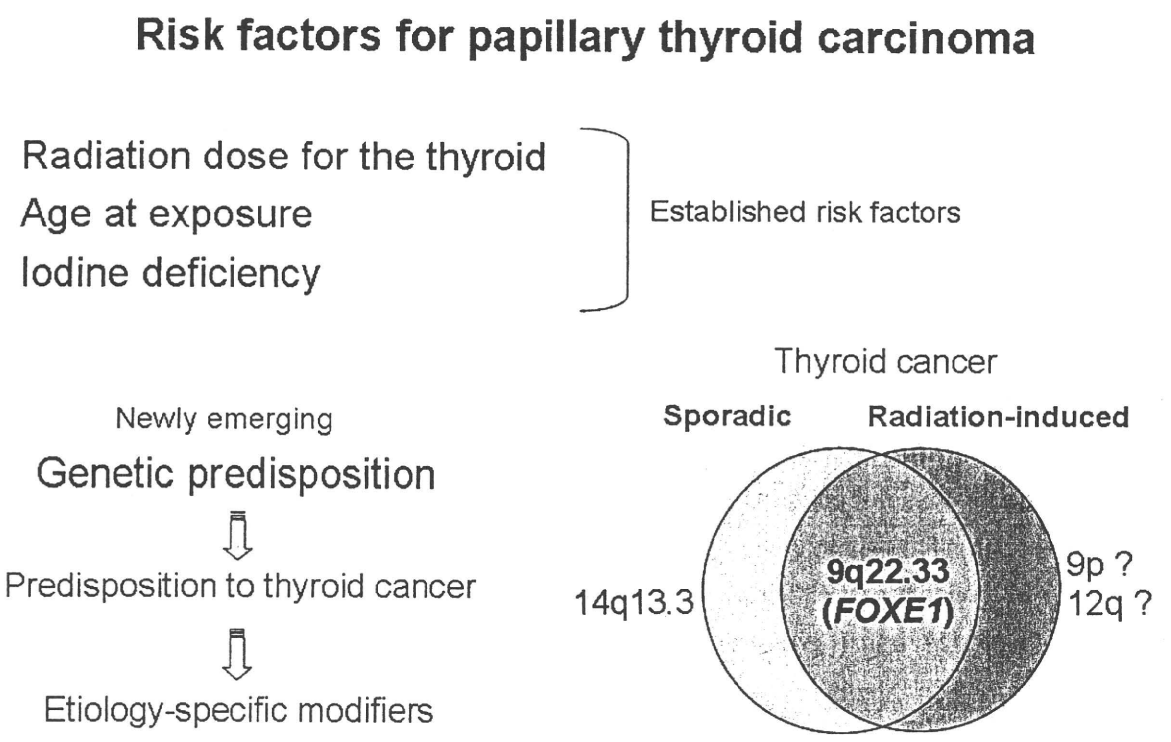

Fig. 2. Genetic predisposition as an emerging risk factor for both sporadic and radiation-induced papillary thyroid carcinoma. Sporadic and radiation-induced PTC share the major genetic determinant of inherited susceptibility to thyroid cancer, *FOXE1* at chromosome 9q22.33, which appears to be stronger than possible etiology-specific genetic markers: on chromosome 14q13.3 (*NKX2-1* or *TTF1*) for sporadic PTC and putative markers on chromosomes 9p and 12q for radiation-induced PTC.

Conclusion

A rapidly growing body of evidence suggests that the identification of molecular "radiation signature" in thyroid cancer is likely to become possible, with certain degree of certainty, in the coming years. The advances in exploring both the damage pattern by genomic microarrays, differential gene expression or immunohistochemically and inherited susceptibility by GWAS and

expression arrays keep on bringing encouraging results yet they are far of being finalized. At present they rather contribute to work out a proof of principle that radiation-induced and sporadic thyroid cancers could be distinguished using a definite set of validated markers. Perhaps this set will include not only the above-mentioned markers as well as essential clinico-pathological information but also other, such as e.g. miRNA and proteomics, whose integration into the spectrum of potential targets and in-depth analyses may enable better insights into the possible classifiers. Its availability will likely allow future personalized cancer risk prediction which is of a significant importance in view of the growing thyroid cancer incidence in the world and also because of the relevance to occupational and expanding medicinal exposures, and radiation emergency medicine issues.

Undoubtedly, Chernobyl cohort is an inestimable source of knowledge in the area. Continuous observation, follow-up and thorough studies are warranted to yield the higher level of understanding. In this regard, international initiatives, such as the Chernobyl Tissue Bank (http://www.chernobyltissuebank.com/) or EC-coordinated GENRISK-T consortium (http://www.helmholtz-muenchen.de/isb/genrisk-t/index.html), Nagasaki University GCOE Program Global Strategic Center for Radiation Health Risk Control (http://www-sdc.med.nagasaki-u.ac.jp/gcoe/projects/index_e.html) and other cooperative efforts would be the principal roadways to solving the problem.

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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 ($mhp = 5.2 \times 10^{-4}$ 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 ($mhp > 3.6 \times 10^{-5}$ for males, $mhp > 1.6 \times 10^{-5}$ 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		Age at diagnosis	
				Range	Mean ± SD	Range	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.

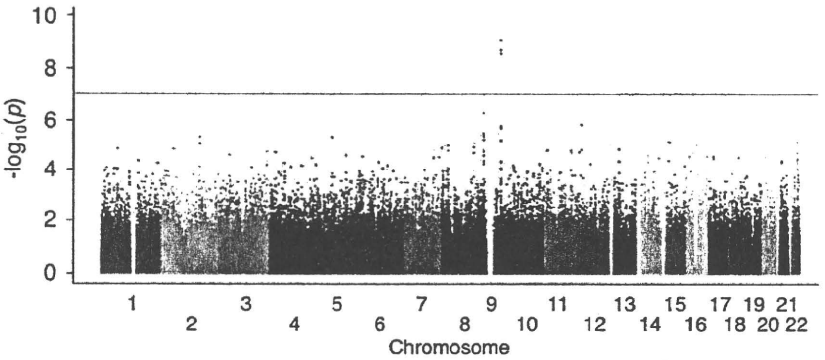

Figure 1. Manhattan plot of the combined GWAS results for Studies 1 and 2. *P*-values calculated by the Trend χ^2 test for 506 840 autosomal SNPs are plotted in $-\log_{10}$ scale with respect to their chromosomal positions. The horizontal line indicates Bonferroni-adjusted $P = 9.6 \times 10^{-8}$.

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.

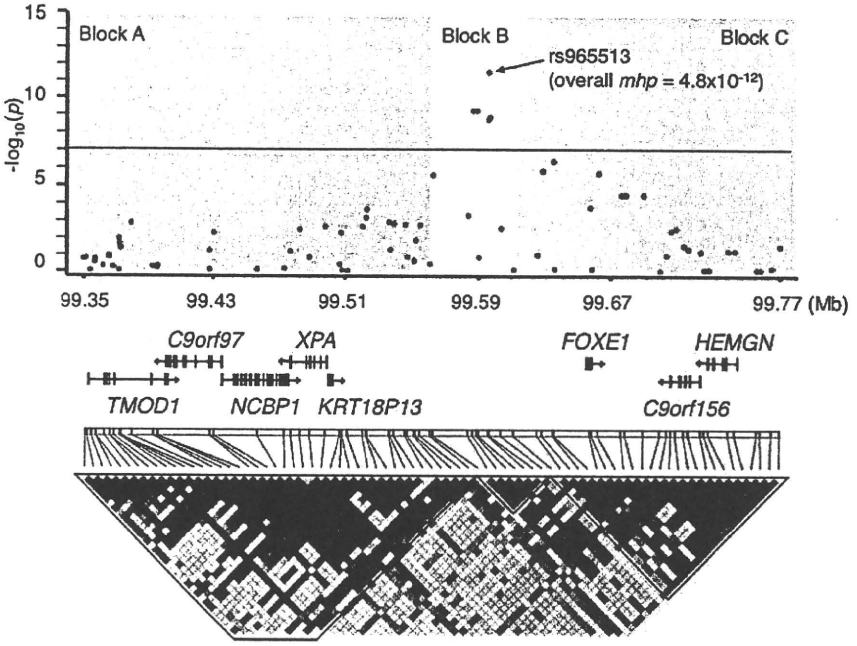

Figure 2. A schematic organization of the human *FOXE1* locus at 9q22.23 with the genome scanning results. Mhp-values calculated by the Trend χ^2 test in $-\log_{10}$ scale were plotted in red circles for SNPs located in the 425-kb region between rs4742698 and rs4618817 at chromosome 9q22.23. The blue 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] Ref.	Var	Chr	Position	Statistics by study Study	Freq var. Case	Control	Trend P^b	OR (95% CI)[c]	Study 1 + 2 Mhp[d]	OR (95% CI)[c]	Study 1 + 2 + 3 Mhp[d]	OR (95% CI)[c]	Gudmundsson *et al.* Freq var. Case	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 ± 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 ± 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 ± SD: 6.8 ± 5.5 years) and 648 controls (mean age ± 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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