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Gene signature of the post-Chernobyl papillary thyroid cancer

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Abstract:	Purpose. Nuclear accident first in Chernobyl and later in Fukushima faced nuclear community with important issues of how to search for and diagnose biological consequences of low dose internal radiation contamination. Although after Chernobyl accident an increase in childhood papillary thyroid cancer (PTC) was observed it is still not clear whether the molecular biology of PTCs that are associated with low dose

	radiation exposure differs from that of sporadic PTC. Methods. We investigated 65 childhood/young adult PTC samples using DNA microarray (Affymetrix, Human Genome U133 2.0Plus) with the aim of identifying molecular differences between radiation-induced (Exposed to Chernobyl Radiation, ECR) and sporadic PTC. All participants were resident of the same region, in order to minimalize genetic- or environmental-related confounding factors. Results. The comparison of gene expression profiles between ECR and non-ECR PTC revealed small but significant difference (global test, $p < 0.01$), with 300 differently expressed probesets ($p < 0.001$) corresponding to 239 genes. Multifactorial analysis of variance showed that besides radiation exposure history, the BRAF mutation exhibited independent effects on PTC expression profile; the histological subset and age of PTC had negligible effects. Ten genes (PPME1, HDAC11, SOCS7, CIC, THRA, ERBB2, PPP1R9A, HDGF, RAD51AP1, CDK1) from the 19 investigated by quantitative RT-PCR were confirmed as being associated with radiation exposure in an independent, validation set of samples. Conclusions. Significant, but subtle, differences in gene expression in the post-Chernobyl PTC are associated with previous low dose radiation exposure.
Response to Reviewers:	Reviewer #1 Thank you for your valuable suggestions. According to the reviewer suggestions Table 1 was simplified and comment on the possible use of this study results in the clinical practice had been added.

Gene signature of the post-Chernobyl papillary thyroid cancer

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Compliance with Ethical Standards

All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards

Declaration of interest

There are no potential conflicts of interest to disclose.

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Abstract

Purpose. Nuclear accident first in Chernobyl and later in Fukushima faced nuclear community with important issues of how to search for and diagnose biological consequences of low dose internal radiation contamination. Although after Chernobyl accident an increase in childhood papillary thyroid cancer (PTC) was observed it is still not clear whether the molecular biology of PTCs that are associated with low dose radiation exposure differs from that of sporadic PTC.

Methods. We investigated 65 childhood/young adult PTC samples using DNA microarray (Affymetrix, Human Genome U133 2.0Plus) with the aim of identifying molecular differences between radiation-induced (Exposed to Chernobyl Radiation, ECR) and sporadic PTC. All participants were resident of the same region, in order to minimalize genetic- or environmental-related confounding factors.

Results. The comparison of gene expression profiles between ECR and non-ECR PTC revealed small but significant difference (global test, $p < 0.01$), with 300 differently expressed probesets ($p < 0.001$) corresponding to 239 genes. Multifactorial analysis of variance showed that besides radiation exposure history, the *BRAF* mutation exhibited independent effects on PTC expression profile; the histological subset and age of PTC had negligible effects. Ten genes (*PPME1, HDAC11, SOCS7, CIC, THRA, ERBB2, PPP1R9A, HDGF, RAD51AP1, CDK1)* from the 19 investigated by quantitative RT-PCR were confirmed as being associated with radiation exposure in an independent, validation set of samples.

Conclusions. Significant, but subtle, differences in gene expression in the post-Chernobyl PTC are associated with previous low dose radiation exposure.

Introduction

Nuclear accident first in Chernobyl and 25 years later in Fukushima faced nuclear community with two important issues - first of how to manage radiation contamination, and second of how to search for and diagnose biological consequences of low dose internal radiation contamination. The biological consequences of radioiodine contamination after Chernobyl accident were observed as early as few years after the accident when an increase in childhood papillary thyroid carcinomas (PTCs) was for the first time demonstrated [1, 2]. Since then, approximately 5000 thyroid cancer cases have occurred in the contaminated regions of Belarus, Ukraine, and Russia with a persisting increased risk of PTC development in irradiated children [3]. Although the increase in PTC incidence in contaminated regions is well demonstrated it is still not clear whether the molecular phenotype of papillary thyroid carcinomas (PTCs) that are associated with low dose radiation exposure differ from that of sporadic PTC.

In small scale molecular studies, when the radiation related thyroid cancers were compared to sporadic ones of similar age, no differences were observed in overall frequency of *RET/PTC* rearrangements, the events crucial for the activation of MAPK cascade [4-12], or in relation to the radiation dose to the thyroid [13]. On the other hand, some other studies reported only distinct types of *RET/PTC* rearrangement in radiation and sporadic cancers [10, 11] or described a difference between radiation-induced and sporadic PTC either by immunohistochemical, genomic, or proteomic approaches [14-16]. However, these results could be biased by many confounding factors [for review see [17]], since except one [15], they were not controlled for the potential impact of genetic and environmental factors, patient's age, histological variant or stage of disease. Such well balanced comparison study was not possible until the foundation of Chernobyl Tissue Bank (CTB). The Chernobyl Tissue

Bank (www.chernobyltissuebank.com), since 1998, has been prospectively collecting samples of thyroid tissue taken from operative specimens from patients aged under 19 at the time of the Chernobyl accident and resident in the contaminated areas of Ukraine and Russia. The prospective nature of the collection means that this now includes patients with thyroid cancer who were born after the radioactive iodine released from the accident had decayed in the environment. Two recent studies, using samples provided by the CTB [18, 19] have reported their results on gene expression phenotype of PTC developing after low dose radiation exposure. However, differences were reported only in normal thyroid tissues [19] or with respect to tumour/normal difference in relation to radiation dose but not as a global difference [20, 18].

In contrast, this study searched for global difference in molecular profile in tumour tissue from patients who were either exposed to the Chernobyl related radiation as children (ECR: exposed to Chernobyl radiation) or were born after January 1, 1987 and therefore were not exposed to radiation (non-ECR; not exposed to Chernobyl related radiation). Both groups resided in the same areas to minimize potential confounding factors (eg. environmental one). Gene expression profile with respect to intrinsic potential confounding factors like age at PTC diagnosis, mutational status, histological subtype of PTC were also investigated. The study was performed within GENRISK-T (EU grant: FP6 36495) consortium the aim of which was to establish whether individual genetic factors influence the risk of developing cancers of the thyroid after exposure to ionizing radiation.

Materials and Methods

The biological material for gene expression analysis was provided by the CTB as aliquots of total RNA from carefully selected PTC samples paired with RNA extracted from their

respective normal thyroid tissue (Supplementary Figure S1), after histopathological review of specimens. After control for RNA and microarray quality 65 PTC samples were analyzed. All biological material was obtained after informed consent from either the patient or his/her guardian, and following an approval of this project by the CTB's External Review Panel (website: www.chernobyltissuebank.com).

The CTB samples were supplemented by 24 tumour samples (Supplementary Figure S1) collected from Polish DTC patients born between January 1, 1987-1994, so were included in non-ECR group (only for the validation and exon array study). All samples were taken during the surgery after the approval by local Ethics Committee and informed consent was obtained from all patients.

I. 3' oligonucleotide microarray study

The study comprised 33 PTC from the ECR group and 32 from the non-ECR group, all obtained from CTB. In the ECR group, the mean thyroid radiation dose was 288 mGy (range 45,4 to 4595). In 7 (21%) patients radiation dose was higher than 1 Gy and only in 5 (15%) lower than 100 mCy. At the time of PTC diagnosis, the ECR patients were slightly but significantly older than the non-ECR. There were no significant differences with respect to the different histological subtypes. The distribution of other factors, especially of disease stage was comparable between both groups (Table 1).

Details on RNA isolation and microarray analysis are provided in Supplementary Material and Methods.

II. Validation QPCR study.

A validation study was carried out on an independent set of tumour samples: 19 independent ECR samples from CTB were compared to 17 Polish non-ECR ones. Since CTB did not posses additional non-ECR tumour samples we decided on Polish ones taking into consideration common ethnicity of Ukrainian and Polish children and stable iodine prophylaxis in Poland after Chernobyl accident that resulted in stable incidence of childhood DTC. Polish samples were selected to ensure their common ethnicity profile with CTB patients. Detailed description of validation group is provided in Table 1.

Details on qPCR analysis are provided in Supplementary Material and Methods.

III. Validation exon array study.

An additional comparison of the exon expression profile was performed for 27 PTC patients under the age of 26 years, 13 ECR and 14 non-ECR (Supplementary Table S1). Twenty RNA samples were derived from PTC patients previously included into the initial 3' microarray study, completed by 7 new PTC cases, amongst them 6 were Polish patients who were born in 1981–1992. Expression analysis of all human exons was carried out using an Affymetrix Human_Exon_1.0_ST_Array.

IV. Data analysis.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [21] and are accessible through GEO Series accession number GSE35570 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35570).

Microarray data were normalized with the GCRMA algorithm. First multi-dimensional scaling was performed. Then, a method of our own was devised for gene filtering based on a

comprehensive analysis of technical accuracy of thyroid cancer and normal thyroid tissue gene expression measurement by oligonucleotide microarrays done in the same samples in 2 independent laboratories at 1'Université libre de Bruxelles, Belgium and in the Institute of Oncology in Gliwice, Poland. Briefly, a subset of 19 CEL files, each done both in Bruxelles and Gliwice laboratory on the same tumour or normal tissues, was compared. This means that the results achieved on the same samples were compared. The overall correlation between pairs of samples by both laboratories were excellent (0.982–0.994). However, while analyzing the transcript-by-transcript correlations, we observed that only a subpopulation of probesets showed excellent reproducibility. There was a trend of rising correlation in conjunction with increasing expression level and variance. After extensive analysis of these relationships, the dataset was subdivided to sets of probesets according to their expression and variance to discriminate between sets with good, acceptable, and poor correlation. Genes showing poor reproducibility (log2 mean expression < 5 and variance < upper quartile of all probe sets variances) were filtered out before the final analysis.

Selection of genes differentially expressed between ECR and non-ECR groups was carried by randomized block design regarding 2 microarrays' batches. We used non-corrected threshold of $p<0.001$. Global test was applied to assess the overall significance of the result, the Benjamini-Hochberg False Discovery Rate (FDR) was calculated for every transcript.

Functional enrichment analysis, including Kyoto Encyclopaedia of Genes and Genomes (KEGG; www.genome.jp/**kegg**/*)* and Panther pathway software (www.pantherdb.org/), were performed to identify metabolic pathways and groups of genes with similar metabolic function based on their annotation. A Bonferroni-corrected P-value 0.05 was considered statistically significant.

Influence of putative confounding factors such as age at PTC diagnosis, presence of solid histoarchitecture, and presence of the *BRAF* or *RET/PTC* alteration, was analyzed by separated three-way analysis of variance (ANOVA) using batches as the fourth, blocking, factor.

Part of the analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html).

Results

Differences between ECR and non-ECR papillary thyroid cancer.

In the unsupervised analysis by multidimensional scaling there was no global change of expression associated with ECR/non-ECR tumour (Figure 1). However, after filtering out the low-reproducibility probesets, with stratification for the 2 batches, 300 probesets were differentially expressed between tumours in ECR and non-ECR patients (non-corrected $p < 0.001$ with FDR for these genes ranging 0.5–8.5%), and globally this difference was significant ($p < 0.01$ in the global test of difference as implemented in BRB Array Tools), i.e., this number of genes was not likely to be obtained by chance. These 300 transcripts corresponded to 239 known genes (Table 2 and Supplementary Table S2 and Figure S2). Pathway enrichment analysis in KEGG database showed that genes differentially expressed between ECR and non-ECR tumours were involved in two endocrine related cancer pathways (prostate and endometrium), non-small cell lung cancer and tight junction. In Panther pathway analysis among others PI3 kinase pathway was involved (Supplementary Table S3).

Analysis of potential confounding factors. In-depth analysis of the potential intrinsic confounding factors, was carried out to exclude their influence on the radiation-related differences in gene expression profile. Initially, age at PTC diagnosis, presence of solid pathomorphology, and presence of the *BRAF* or *RET/PTC* alteration known to trigger PTC were considered for their relation with ECR/non-ECR differences by separate three-way analyses of variance (Supplementary Table S4). No association of gene expression profile with patient age (younger than 16 years of age vs older) was seen at FDR < 10%. *BRAF* mutation was significantly associated with the PTC gene expression profile (794 probesets), while there were only 13 probesets associated with *RET/PTC* rearrangement with the same criteria. The gene expression difference related to radiation exposure was also independently significant in the presence of solid pathomorphology. In the final analysis of putative confounding factors, we included *BRAF* mutation and solid PTC variant with radiation exposure. Our analysis revealed that radiation exposure was associated with differences in gene expression regardless of *BRAF* mutation effect (significantly associated with a number of transcripts) and of the influence of solid PTC variant, that was negligible in multivariate analysis at the FDR < 10% criterion (Table 3).

Validation of the results by qPCR. To validate the low-dose irradiation-induced changes in gene expression, we selected 19 genes from the ECR/non-ECR gene signature for qPCR on an independent set of 36 PTC. In the ECR group there were 19 PTC samples, derived from CTB and independent from microarray set, and in the non-ECR group 17 samples, collected in Poland, derived from adolescent patients operated because of PTC (Supplementary Figure S1). The gene selection was performed, based on preliminary microarray analysis (data not shown). The criterion for selection was the significant difference in expression between ECR and non-ECR, and biological function of the gene: we decided to select genes involved in response to DNA damage. The curated list of 19 genes was selected (Supplementary Table

S5) and expression of all of them was estimated by QPCR in independent from set of PTC samples, separate from those investigated by microarray. Ten of them (52%) were validated: *PPME1* (fold changes of 1.19× and 1.25× in the ECR group in the microarray experiment and in qPCR validation, respectively), *HDAC11* (fold changes of 1.47× and 1.25×), *SOCS7* (fold changes of 1.38× and 1.22×), *CIC* (fold changes of 1.26× and 1.22×), *THRA* (fold changes of 1.32× and 1.16×), *ERBB2* (fold changes of 1.32× and 1.34×), *PPP1R9A* (fold changes of 1.5× and 1.23×), *HDGF* (fold changes of 1.19× and 1.28×), *RAD51AP1* (fold changes of 0.58× and 0.55×) and *CDK1* (fold changes of 0.57× and 0.67×) (Figure 2). Genes that were not confirmed in the qPCR analysis included: *MKNK2, RAS, JUB, USP15, FAM105A, MNT, GPX7, PALM3, GNA11.*

Validation by exon array. Finally, 27 PTC (13 ECRs, and 14 non-ECR) were considered for the exon expression validation analysis (in the non-ECR group there were 8 CTB-derived PTC samples and 6 Polish samples). From the 239 genes specified by the initial gene expression microarray study, 52 (22%) were confirmed at the level of FDR < 10% (Table 2 and Supplementary Table S2).

Discussion

Although the rise of thyroid cancer incidence after Chernobyl is evident [3, 22], the question of the potential molecular peculiarity of these induced tumours has not yet been resolved. Answering this question is not only of scientific interest, but also may expand our knowledge on how to manage internal radiation contamination.

In our study on post Chernobyl papillary thyroid cancer we observed small but significant changes in expression of 239 genes ($p < 0.01$) between tumours arising after exposure to low-dose radiation after Chernobyl accident, and sporadic PTCs. Our study is among the first to find the differences in gene expression profiles between radiation-induced and sporadic PTC in patients matched for their ethnicity, place of living, sex, histopathology, disease stage, and age of diagnosis. Five previous transcriptomic studies comparing radiation-induced and sporadic thyroid cancer [23-25, 16, 26] were limited by the small number of studied cases, and were far from matched between sporadic and radiation-induced PTC due to differences in geographical distribution of cases [23, 25, 26], in PTC stage, [25] as well as comparing expression alterations in radiation-induced cancer to data repositories of sporadic PTC in adults [16]. The recently published study by Abend et al. [20], that analyzed a well characterized cohort of radiation induced PTC, showed radiation dose dependent gene expression changes, but did not globaly compare exposed and not exposed PTC. Our results support their general conclusion on the long term differential gene expression in PTC arising after ionizing radiation exposure. This observation is also supported by recent results of [15] who demonstrated that PTC driver alterations are more prevalent in PTC in children that had been exposed to radiation.

Although to our knowledge our matched group of radiation exposed and sporadic PTC is optimal with currently available biological samples we are aware of potential drawback, in the ability to identify sporadic PTC developing in radiation exposed patients. According to epidemiological estimation about 29% in our ECR group may have developed PTC in the absence of radiation exposure [27]. The figure may possibly be even higher if the increased identification of PTC due to screening of the population is taken into account. We therefore cannot rule out admixture of some sporadic and radiation induced cancers in our ECR group. However, we were able to identify significant, although subtle, differences in gene expression

profile between ECR and non-ECR cancers. We can speculate that admixture of sporadic cases is one of the reason for very subtle difference in gene expression ranging from 0.48–3.42. Also on molecular level we failed to separate within ECR group tumours clustering closer to non-ECR group neither in PCA (Figure 1) nor in more detailed supervised analysis. This fact leads us to speculate that the different expression between ECR and non-ECR tumours is rather related to radiation response than to carcinogenesis.

Our negative findings using PCA are in line with results of Dom et al. [19], who in cooperation and in parallel with our group studied gene expression in normal thyroid tissue of radiation exposed and not exposed patients. They also were not able to show any difference in PCA and only SAM analysis adjusted for age was able to identify 403 differentially expressed genes in normal thyroid tissues in their analysis. Similarly in our study the difference between ECR and non-ECR tumours was only possible to be detected after careful quality assurance, including gene filtering according to their expression level and variance. Thus, with such a stringent criterion, it is not surprising that there were only few overlapping genes when we compared our 239 differentiating genes with the results of others. None of the top 15 candidate genes found to be differing between radiation-induced and sporadic PTC by Port et al. [25] overlapped with ours or 2 other sets. No overlap was either found for the 10 genes, validated by us by qPCR. Only 1 (*NEDD4L*) identified by Detours [23], 4 (*ALDH6A1*, *TPD52L1, GPX1, ECE1*) identified by Stein et al. [16] and 2 (*MYO1C*, *IGF1R*) identified by Ugolin et al. [26] series of genes were observed in our microarray gene signature. Given that our multi-factorial analysis of variance excluded the contribution of age differences and tumour pathology in gene expression profiles between the ECR and non-ECR group, one can hypothesize that the genes identified here reflect a true difference between non-ECR and ECR related PTC. However, our results also support an independent effect on PTC gene expression profile by the presence of a *BRAF* mutation. Interestingly, the effect the of the presence of

RET/PTC rearrangements was smaller [28]. This is consistent with previous studies showing the difference between *BRAF* and *RET/PTC* impact on gene expression of thyroid cancer [29, 30]. Contrary to previously reported incidence of post-Chernobyl PTC [7, 8, 12, 31], the frequency of *RET/PTC* was not as high, and of *BRAF*, not as low. This is also consistent with the fact that the median age of patients was 17.7 years, which is distinctly higher than in previous post-Chernobyl cohorts [3], but similar to recently reported by Sassolas et al. [32]. The relationship between age at diagnosis and frequency of *BRAF* and *RET/PTC* alterations has also been previously identified in Ukrainian patients [33]. The requirement to age match with sporadic cases of PTC, which is more common in older children, for this study meant that patients in the ECR group were also slightly older than in the previous studies that did not use age-matched controls. In addition, 52 of our genes were validated by exon array analysis done in the partially independent and smaller set of tumours.

Environmental factors, such as differences in iodine deficiency also need to be taken into consideration [34]. However in our study cases from ECR and non-ECR group were evenly distributed within different regions (oblast) of residence and to our best knowledge in a retrospective series cases this is the best available method to control for differences in iodine dietary status. Unlike other authors [20, 18] we did not show formal analysis of gene expression in relation to individual radiation doses provided by CTB [35]. Although Spearman dose-response correlation indicated a few significant genes (data not shown), due to uncertainty of radiation dose and possible admixture of sporadic cases in non-ECR group we consider these data too much biased. Furthermore, the recently published studies indicate more diversified gene expression profile at decreasing absorbed dose. This observation was in the mouse thyroid cell after injection of different amounts of the 211 At or 131 I radionuclide [36, 37]. It was hypothesized that at high absorbed doses, the DNA lesions might have been

too complex to be properly repaired, resulting in reduced cellular response compared to the lower absorbed doses.

The important feature of the investigated PTC was the young age of their hosts, which contributed to the different PTC gene expression profile when compared to adult patients (data not shown). However, our radiation gene signature contained both genes, which did and did not contribute to the tumour/normal difference in the studied patients (data not shown) [38, 39]. Thus, our paper defines the difference in gene expression related to radiation exposure, the functional consequences of this need to be defined. To understand the underlying biological mechanisms, the genes confirmed by qPCR need to be examined in independent PTC cases in relation to G2/M cell cycle arrest. The simultaneous lower expression of *CDK1* and *RAD51AP* may represent impaired repair of the radiation-induced DNA damage in ECR patients. The expression of *CDK1* in fibroblasts is reduced in response to radiation [40], and its suppression is essential for DNA damage-induced G2 arrest [41]. *CDK1* is required for efficient 5′ to 3′ resection of double-strand break ends, and for the recruitment of both the single-stranded DNA-binding complex, RPA, and the RAD51 recombination protein [42]. Decreased *RAD51AP*, encoding an enhancer of *RAD51*, observed in tumours from ECR patients is consistent with this suggestion as genetic ablation of *RAD51AP1* leads to enhanced sensitivity to chromosome aberrations upon DNA damage [43]. *RAD51AP1*-depleted cells have deficits in recombination-based repair of a DNA double-strand break, and exhibit chromatin breaks both spontaneously and upon DNA-damaging treatment [44]. The simultaneous increase of expression of *HDAC11* in ECR-related PTC creates a link to transcriptional repression and epigenetic landscaping [45], and can be interpreted as concordant with both *CDK1* and *RAD51AP1* decrease as the latter is regulated by E2F family of transcription factors, while histone deacetylases interact with RB-E2F to inhibit gene transcription and are activated by radiation [46]. This effect may be stronger at

the basic higher expression level. The reduced expression of *PPME1* may also be related to the repair of gamma-irradiation-induced DNA damage, which is regulated not only by PP1, but also by PP2A phosphatase inhibition [47]. Its protein product, protein phosphatase methylesterase 1, is regarded as a key molecule that sustains, via inhibition of PP2A, the activation of ERK activity in cancer cells [47, 48]. The higher expression of this gene group in thyroid cancers of ECR group may lead to the higher activation of MAP cascade downstream of growth factors, but upstream of RAF and facilitate neoplastic transformation towards papillary thyroid cancer [10]. In fact, 4 out of 14 genes known to modulate *PP2A* were significantly changed in ECR-related tumours (Table S2). These effect can be further enhanced be up-regulation of *ERBB2* and THRA (thyroid hormone receptor A) in ECR group. Recently *THRA*-rs939348 was confirmed a risk factor for DTC [49] and one may speculate that its increased expression in ECR tumours is a persistent response to radiation DNA damage which may cooperate with other genes in DTC development.

Obviously, a number of other potential speculative explanations for observed gene expression differences could be presented. It cannot be excluded that cancer induced by a single dose of radiation shows a difference in cellular homogeneity (increased number of multiplied transformed cells and their desynchronization), kinetics of progression, or even to the tumour size at diagnosis.

Recently important paper related to molecular biology of thyroid cancer discussing the results of The Cancer Genome Atlas (TCGA) has been published [28]. The results of the study indicated the relatively low number of novel genomic events in PTC compared to the previous knowledge and indicated the presence of subtypes, mainly related the type of initiating somatic abnormality. It is an obvious next step to apply genomic sequencing to analyze in-depth the association of these subtypes and heterogeneity related to different initiating

mutations with the profile of radiation-induced PTC. Noteworthy, the expression of all genes, characteristic for ECR PTCs according to our signature, in PTCs investigated by TCGA was high.

The important question arises whether subtle differences between the profile of radiation-induced and sporadic PTC have any clinical significance. Probably they reflect not the profound differences in the underlying disease, but rather the different disease kinetics, cellular composition or – most interestingly – additional molecular mechanisms operating on in the radiation-induced cancer. The proposed classifier is not sufficient itself to distinguish the cancers induced by low-dose radiation from sporadic cases, and our results indicate that the effect of radiation is similar in scale to many other factors influencing the variability of gene expression in PTC. We did not find any gene expression differences profound enough to influence the clinical course of the disease, and this is in line with the clinical observations indicating the similar prognosis in radiation-induced post-Chernobyl childhood PTC [3, 50)] However, we interpret the differences observed by us as the excellent starting point to assess the importance of genes constituting the radiation signature in the pathogenesis of PTC.

In conclusion, we report significant, but subtle, differences in gene expression in the post-Chernobyl PTC that are associated with low dose radiation exposure. Since the population exposed to low dose thyroid radiation (either medical or accidental) is increasing the study can serve as a basis for further studies on thyroid low dose radiation susceptibility.

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Figure Legends

Figure 1.

Multi-dimensional scaling of samples. Samples coloured on red are ECR tumors, green are non-ECR tumors, blue are ECR normal thyroids and cyan are nECR normal thyroids

Figure 2.

Genes validated in qPCR study.

Table 1**. Comparison of ECR and non-ECR groups** used in the microarray study and in an independent qPCR validation study. Histopathology was evaluated in CTB according to following criteria (1) pure classic PTC; (2) *PTC with follicular areas (denotes dominant pattern of follicular structures); (3) **PTC with solid areas (denotes dominant pattern of solid areas). In QPCR validation study (non ECR group from Poland) PTC histopathology was evaluated according to WHO 2004 criteria.

	Microarray study set			**QPCR validation set**			**Statistical comparison microarray to validation set**	
	ECR	non-ECR	Statistical compa-rison; p- level	ECR	non-ECR	Statistical compa-rison; p- level	ECR; p- level	non-ECR; p- level
Number	33	32	not done	19	17	not done	not done	not done
Female/Male	23/10	26/6	ns	14/5	14/3	ns	ns	ns
Age at exposure; median (years)	2.3 (0.1-8.3)	not done	not done	2 (0.5-11.2)	not done	not done	ns	not done
Age at diagnosis; median (years)	17.7 (14.7-24.5)	16.3(7.7-21)	**0.0002**	19.5 (1.3-23,9)	17.4 (11.6-21.5)	0.06	0.07	**0.04**
				Place of residence (oblast)				
Kiev	10 (30.3%)	12 (37.5%)	Ns	**7** (36%)	Poland		ns	not done
Zhytomir	8 (24.2%)	5 (15.6%)	Ns	5 (26%)	Poland		ns	
Chernigow	8 (24.2%)	5 (15.6%)	Ns	6 (31.5)	Poland		ns	
Sumy, Rovno, Chercassy, Pipriad	7 (21.2%)	10 (32.3%)	Ns	1 (5%)	Poland		ns	
				Histopathology				
Pure classic PTC	4 (12%)	7 (22%)	ns	4 (21.1%)	12/17 (70.6%)	not done	ns	not done
with follicular areas*	17 (52%)	11 (33%)	ns	6 (31.6%)	5/17 (29.4%)	not done	ns	not done
PTC with solid areas	12 (36%)	14 (43%)	ns	8 (42%)	0	not done	not done	not done
Unknown	0	0	not done	1/19 (5.2%)	0	not done	not done	not done

Mutational status of PTC							
RET/PTC rearrangements (+)	10 (30.3%)	8 (25%)	ns	not available			not done
RET/PTC rearrangements (-)	19 (57.6%)	22 (66.7%)	ns				
RET/PTC status unknown	4 (12.1%)	2 (6.3%)	ns				
BRAF V600E mutation (+)	8/33 (24.2)	6/32 (18.8%)	ns				
BRAF V600E mutation (-)	22 (66.7%)	21 (65.6%)	ns				
BRAF V600E mutation unknown	3 (9.1%)	5 (15.6%)	ns				
Primary tumour (T stage)							
1	12 (36%)	11 (34%)	ns	3 (15.8%)	8 (47.1%)	ns	ns
2	4 (12%)	5 (16%)	ns	4 (21.1%)	4 (23.5%)	ns	ns
3	17 (52%)	16 (50%)	ns	11 (57.9%)	5 (29.4%)	ns	ns
Unknown	0	0	-	1/19 (5.2%)	0	-	
Lymph nodes (N stage)							
0	14 (42%)	14 (34%)	ns	8 (42.1%)	6 (35.3%)	ns	ns
1	19 (58%)	18 (56%)	ns	10 (57.9%)	11 (64.7%)	ns	ns
1a	12 (36%)	8 (25%)	ns	7 (36.9%)	4 (23.5%)	ns	ns
1b	7 (22%)	10 (31%)	ns	3 (15.8%)	7 (41.2%)	ns	ns
Unknown	0	0		1	0		
Distant Metastases (M stage)							
0	29 (87%)	30 (94%)	ns	17 (89.3%)	16 (94.1%)	ns	ns
1	4 (13%)	2 (6%)	ns	2 (10.7%)	1 (5.9%)	ns	ns

ns - not significant

Table 2. Top 30 genes differentiating ECR and non-ECR papillary thyroid cancers.

		(a)		(b)		(c)		(d)	
	Gene	**ECR/non-ECR expression microarray**		**ECR/non-ECR 3-ANOVA**		**ECR/non-ECR exon microarray**		**ECR/non-ECR QPCR**	
Gene symbol	Description	FDR	Fold change	P-value	FDR	FDR	Fold change	FDR	Fold change
USP27X	ubiquitin specific peptidase 27, X-linked	0,00516	1,34	0,0000093	0,05	Ns		Nd	
ANKS6	ankyrin repeat and sterile alpha motif domain containing 6	0,0103	1,34	0,0000152	0,05	0,058	1,35	Nd	
GPX7	glutathione peroxidase 7	0,0103	0,61	0,0000007	0,019	9		Ns	
MNT	MAX binding protein	0,0187	1,28	0,0000031	0,04	0,058	1,22	Ns	
PPP1R9A	protein phosphatase 1, regulatory (inhibitor) subunit 9A	0,0341	1,5	0,0000137	0,05	Ns		0,021	1,225
MKNK2	MAP kinase interacting serine/threonine kinase 2	0,0341	1,29	0,000114	0,094	0,133	1,22	Ns	
DDR1	discoidin domain receptor tyrosine kinase 1	0,0341	1,34	0,000353	Ns	0,070	1,33	Nd	
HNRNPUL2	heterogeneous nuclear ribonucleoprotein U-like 2	0,0341	1,25	0,0000225	0,053	0,070	1,08	Nd	
GNL1	guanine nucleotide binding protein-like 1	0,0341	1,23	0,000235	Ns	0,101	1,12	Nd	
PTCD3	Pentatricopeptide repeat domain 3	0,0341	0,8	0,0000205	0,053	Ns		Nd	
ZBTB43	zinc finger and BTB domain containing 43	0,0341	1,4	0,000068	0,09	Ns		Nd	
CIC	capicua homolog (Drosophila)	0,0341	1,26	0,0000521	0,09	Ns		0,008	1,221
GMEB2	glucocorticoid modulatory element binding protein 2	0,0341	1,26	0,000213	Ns	0,070	1,18	Nd	

ZBTB7C	zinc finger and BTB domain containing 7C	0,0341	1,44	0,00015	Ns	0,148	1,22	Nd	
KIAA0182	KIAA0182	0,0341	1,32	0,000311	Ns	0,123	1,14	Nd	
GNA11	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	0,0341	1,25	0,000112	0,094	0,130	1,22	Ns	
HDAC11	histone deacetylase 11	0,0341	1,47	0,000071	0,09	0,114	1,20	0,004	1,247
SPATA2L	spermatogenesis associated 2-like	0,0341	1,26	0,000695	Ns	Ns		Nd	
SLC25A23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23	0,0341	1,41	0,000126	Ns	0,090	1,31	Nd	
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	0,0341	0,73	0,0000142	0,05	Ns		Nd	
PALM3	Paralemmin-3	0,0341	3,42	0,000182	Ns	0,136	1,17	Ns	
LYPLA2	lysophospholipase II	0,0341	1,23	0,000138	Ns	Ns		Nd	
MOB2	Mps one binder kinase activator-like 2	0,0341	1,22	0,000189	Ns	Ns		Nd	
HDGF	hepatoma-derived growth factor (high-mobility group protein 1-like)	0,0349	1,19	0,0000155	0,05	0,070	1,13	0,021	1,275
GOPC	golgi-associated PDZ and coiled-coil motif containing	0,0359	1,36	0,0000616	0,09	Ns		Nd	
JUB	jub, ajuba homolog (Xenopus laevis)	0,0359	1,39	0,000181	Ns	0,093	1,25	Ns	
CTBP2	C-terminal binding protein 2	0,0359	1,29	0,000171	Ns	Ns		Nd	
EHMT2	euchromatic histone-lysine N-methyltransferase 2	0,0362	1,27	0,00011	0,094	0,101	1,17	Nd	
RAD51AP1	RAD51 associated protein 1	0,0362	0,58	0,000218	Ns	0,100	0,86	0,021	0,553

SPRYD3	SPRY domain containing 3	0,0362	1,3	0,000672	Ns	0,075	1,32	Nd

Table 3. Gene signature of Exposure to Chernobyl Radiation: analysis of putative confounding factors.

First, 4 different 3-way analyses were performed (with series-related subgroups), for interaction with age, with the presence of *RET*, of *BRAF*, and with solid histoarchitecture. For each of the analyzed factors, the number of genes significant at $p < 0.001$ was shown in the Web-Supplement Table S4. A final analysis, performed for the 3 factors with the strongest effect and 2 series of examination, which included Exposure to Chernobyl-related Radiation, *BRAF* mutation, and pathology (with the subdivision into 2 groups, one joining classic and follicular variant, and the second both specified subgroups with solid appearance) is shown.

Effect	No of probesets at $p < 0.001$	No of probesets at FDR < 10%
Exposure to Chernobyl-related radiation	196	33
BRAF mutation	183	114
Pathology classical and follicular/solid component	32	0

Figure 1. Multi-dimensional scaling of samples. Samples coloured on red are ECR tumors, green are non-ECR tumors, blue are ECR normal thyroids and cyan are nECR

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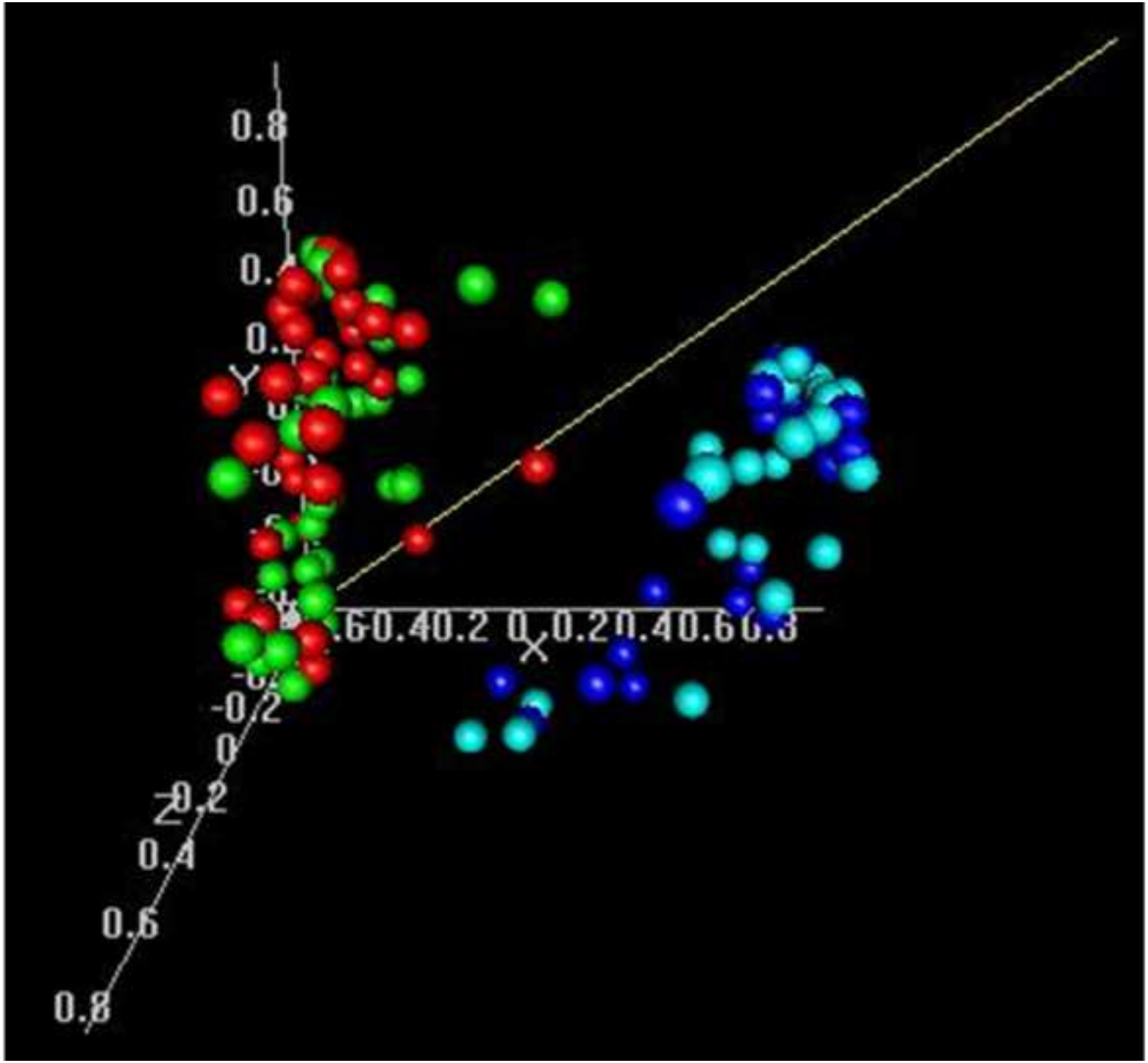

Figure 2. Genes validated in qPCR study.

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