RADIATION RESEARCH **183**, 249–261 (2015)
0033-7587/15 $15.00

DOI: 10.1667/RR13758.1

Association of Radiation-Induced Genes with Noncancer Chronic Diseases in Mayak Workers Occupationally Exposed to Prolonged Radiation

Michael Abend,[a,1] Tamara Azizova,[b] Kerstin Müller,[a] Harald Dörr,[a] Sven Doucha-Senf,[a] Helmut Kreppel,[c] Galina Rusinova,[b] Irina Glazkova,[b] Natalia Vyazovskaya,[b] Kristian Unger,[d] Herbert Braselmann[d] and Viktor Meineke[a]

[a] *Bundeswehr Institute of Radiobiology affiliated to the University of Ulm, 80937 Munich, Germany;* [b] *Southern Urals Biophysics Institute (SUBI), Russian Federation, Ozyorsk 456780, Russia;* [c] *Bundeswehr Medical Office, Department IX 1, CBRN Med Defence, 80937 Munich, Germany; and* [d] *Research Unit of Radiation Cytogenetics, Integrative Biology Group, Helmholtz-Zentrum München, 85764 Neuherberg, Germany*

Abend, M., Azizova, T., Müller, K., Dörr, H., Senf, S., Kreppel, H., Rusinova, G., Glazkova, I., Vyazovskaya, N., Unger, K., Braselmann, H. and Meineke, V. Association of Radiation-Induced Genes with Noncancer Chronic Diseases in Mayak Workers Occupationally Exposed to Prolonged Radiation. *Radiat. Res.* 183, 249–261 (2015).

We examined the association of gene expression with noncancer chronic disease outcomes in Mayak nuclear weapons plant workers who were exposed to radiation due to their occupation. We conducted a cross-sectional study with selection based on radiation exposure status of Mayak plant workers living in Ozyorsk who were alive in 2011 and either exposed to: combined incorporated Plutonium-239 (^{239}Pu) and external gamma-ray exposure (n = 82); external gamma-ray exposure alone (n = 18); or were unexposed (n = 50) of Ozyorsk residents who provided community-based professional support for plant personnel and who were alive in 2011. Peripheral blood was taken and RNA was isolated and then converted into cDNA and stored at −20°C. In a previous analysis we screened the whole genome for radiation-associated candidate genes, and validated 15 mRNAs and 15 microRNAs using qRT-PCR. In the current analysis we examined the association of these genes with 15 different chronic diseases on 92 samples (47 males, 45 females). We examined the radiation-to-gene and gene-to-disease associations in statistical models stratified by gender and separately for each disease and exposure. We modeled radiation exposure as gamma or ^{239}Pu on both the continuous and categorical scales. Unconditional logistic regression was used to calculate odds ratios (OR), 95% confidence intervals (CI), and the concordance for genes that were significantly associated with radiation exposure and a specific disease outcome were identified. Altogether 12 mRNAs and 9 microRNAs appeared to be significantly associated with 6 diseases, including thyroid diseases (3 genes, OR: 1.2–5.1, concordance: 71–78%), atherosclerotic diseases (4 genes, OR: 2.5–10, concordance: 70–75%), kidney diseases (6 genes, OR: 1.3–8.6, concordance: 69–85%), cholelithiasis (3 genes, OR: 0.2–0.3, concordance: 74–75%), benign tumors [1 gene (AGAP4), OR: 3.7, concordance: 81%] and chronic radiation syndrome (4 genes, OR: 2.5–4.3, concordance: 70–99%). Further associations were found for systolic blood pressure (6 genes, OR: 3.7–10.6, concordance: 81–88%) and body mass index [1 gene (miR-484), OR: 3.7, concordance: 81%]. All associations were gender and exposure dependent. These findings suggest that gene expression changes observed after occupational prolonged radiation exposures may increase the risk for certain noncancer chronic diseases.

INTRODUCTION

For many years there has been ongoing debate about whether past exposure to ionizing radiation leaves a unique permanent signature in the genome (*1*). Discovery of such a signature would have a strong impact on epidemiological and radiobiological studies since it would have the potential to discriminate between radiation-related diseases and sporadic diseases in humans or animal models and subsequently, the process of radiation carcinogenesis might be better understood (*2*). Recent studies by Hande *et al*. and Mitchell *et al*., using the sophisticated chromosome labeling technique (mBand and mFISH), suggested that interchromosomal aberrations (*3, 4*) and complex chromosome aberrations (*2*) in lymphocytes persist in individuals many years after occupational exposure to densely ionizing radiation. These techniques were applied to healthy former nuclear weapons workers who were occupationally exposed from 1949 onward at the Mayak Production Association (PA) near Ozyorsk, Russia (*5, 6*). The radiation workers were employed either in plutonium manufacturing/processing facilities or in a nuclear reactor facility. The plutonium workers but not the reactor workers were exposed to densely ionizing alpha particles as a consequence of plutonium inhalation.

Editor's note. The online version of this article (DOI: 10.1667/RR13758.1) contains supplementary information that is available to all authorized users.

[1] Address for correspondence: Bundeswehr Institute of Radiobiology affiliated to the University of Ulm, Neuherbergstr. 11, 80937 Munich, Germany; e-mail: michaelabend@bundeswehr.org.

In our recently published work, we performed a gene expression study of a group of Mayak workers who were exposed either to combined internal alpha particles due to incorporated Plutonium-239 (^{239}Pu) and external gamma rays, or to external gamma rays only. Peripheral blood was taken from workers older than 70 years of age, suffering from 29 different chronic diseases such as atherosclerotic diseases or diabetes. For that study, we screened the whole genome for radiation-associated candidate genes from this cohort and validated 25 mRNAs and 20 miRNAs (*7, 8*). Within this validation step we employed another methodology (quantitative real time PCR, qRT-PCR), used 92 different blood samples and adjusted each of our radiation-to-gene models for 26 potential confounders. These confounders were: age at exposure, age at biosampling, demographic data (education, ethnicity, birth date, gender), social habits (smoking and alcohol), data related to health status [diastolic/systolic blood pressure, body mass index (BMI, defined as weight in Kg divided by height in meters squared)], diagnosis of benign (mostly skin) tumors as well as noncancer chronic diseases (chronic radiation syndrome, atherosclerosis, fibroadenoma of breast, cholelithiasis and others). We then examined whether significant dose-to-gene relationships remained after considering all potential confounders. We added these 26 confounders separately to all models and for each exposure type (analytically we discriminated between external gamma or internal ^{239}Pu even in the individuals exposed to combined external gamma and ^{239}Pu) and analyzed whether the dose-to-gene associations remained. When adding each of these confounding factors to our ''basic'' models we found that: 1. Confounders, such as age at blood sampling, smoking, alcohol and BMI, were not significantly associated with the models and, therefore, not included; 2. Confounders were significantly associated with the models and significant exposure-to-gene associations became insignificant. In the latter case the gene was excluded from further analysis, thus reducing the number of candidate mRNAs and miRNAs after controlling for potential confounder. Ultimately, 15 mRNA and 15 miRNAs still showed statistically significant dose-to-gene relationship even after adjustments for all 26 confounders (*7, 8*).

In this current study, we sought to determine the clinical significance of altered gene expression after chronic radiation exposure in our validated 15 mRNAs and 15 miRNAs. Specifically, we tested the association between altered gene expression intensity and risk of chronic diseases other than cancer. We then examined the association of our pre-identified radiation-associated genes with the 29 different chronic diseases from which the Mayak workers and Ozyorsk residents study were suffering.

MATERIALS AND METHODS

Study Population

The Mayak Production Association, which is located near the city of Ozyorsk in the Southern Urals in the Russian Federation, began operations in 1948 as the first and largest nuclear weapons facility in the former Soviet Union. The study cohort included all workers first employed in one of the main plants of the Mayak PA (i.e., the reactor, radiochemical and plutonium plants) from the start of operations up until the end of 1958, independent of gender, age, nationality, occupation and other characteristics [see ref. (*6*)]. Workers who were involved in incidents/accidents and developed acute radiation syndrome, as well as workers exposed to radionuclides other than ^{239}Pu, such as tritium, were excluded from the study cohort. Doses from other potential sources of exposure, including neutron exposures to workers of reactors and the plutonium plant and diagnostic medical X-ray examinations of workers (*16*), were negligible compared with the occupational photon and plutonium doses received by cohort study members (*6*). The cohort included 12,210 individuals (and 9,387 living in Ozyorsk), of whom 3,552 (29.1%) were women. More than half of these workers (68.2%) were employed during the first 6 years of operation at Mayak PA [1948–1953 (*6*)]. The mean age of the workers at the start of their employment at Mayak PA ($\pm$SD) was 25.1 $\pm$ 7.4 years for men and 24.6 $\pm$ 6.0 years for women. The mean duration of work ($\pm$SD) at the reactors, and the radiochemical and plutonium plants was 14.4 $\pm$ 13.3, 11.5 $\pm$ 12.96 and 13.9 $\pm$ 12.6 years, respectively (*6*). Workers at the reactors were potentially exposed to external gamma rays, whereas workers at the radiochemical and plutonium plants, in addition to radiation from external sources, could be exposed to alpha-particle radiation from internally deposited ^{239}Pu. Of 9,387 members of Mayak PA worker cohort living in Ozyorsk, 2,093 individuals exposed solely to external gamma rays absorbed a total whole-body dose $>$0.5 Gy. This gamma dose represented the inclusion criteria for individuals who were eligible for the current study. For individuals who experienced combined occupational exposures, total whole-body doses from external gamma rays had to exceed 1.01 Gy and the plutonium body burden had to be $>$0.7 kBq to be eligible for the current study (Fig. 1). The unexposed group comprised Ozyorsk residents who worked in the community in supportive vocations (e.g., teachers and doctors) but were not employees of Mayak PA. They were never exposed occupationally, never involved in any cleanup operations following radiation accidents and never resided in contaminated areas. Biophysical examinations of unexposed individuals (comparison subjects) revealed no plutonium in their bodies. Only Ozyorsk residents with known vital status (May–June 2011, n=2,048), who were living in Ozyorsk and were alive as of 2011 (n = 454), were included in our study to better control for undesired local differences. From these 454 Mayak PA workers, we requested blood samples during their follow-up in 2011. Based on availability of blood samples in 2011 and our inclusion/exclusion criteria we selected 18 Mayak workers exposed to external gamma rays only, 82 exposed to alpha particles due to plutonium incorporation and external gamma rays and 50 unexposed Ozyorsk residents serving as comparison subjects (Fig. 1). Blood samples were sent from Russia to the Bundeswehr Institute of Radiobiology and processed. Eighteen samples were lost during the RNA isolation process and 40 samples were used for screening purposes to identify candidate genes in phase I, leaving 92 blood samples (obtained from 47 males and 45 females) eligible for validation in phase II (Fig. 1) (*8*). Using these samples during this study we examined the association of validated candidate genes with the frequency of altogether 29 chronic diseases and parameters related to their health status (systolic/diastolic blood pressure, BMI) in phase III. Every cohort member underwent medical follow-up and disease diagnostics were conducted on a regular basis according to a specially developed standard program (*6*). All workers, including the unexposed individuals, were examined uniformly irrespective of their exposures. Diseases and subgroups were coded by clinicians according to the International Classification of Diseases, Ninth Revision (ICD-9). In our gender stratified groups (comprising up to 47 males and 45 females), there were occasions where less than 10 individuals suffered from a subgroup of 14 out of the 29 total diseases, i.e., cancer,

Inclusion/exclusion criteria [Mayak Production Association (PA) cohort]	External γ ray only	External γ ray and internal Pu	N	
• No. of Ozyorsk residents who worked at Mayak PA	3,803	5,584	9,387	
• Total dose >0.5 Gy/>1.01 Gy + >0.7 kBq[1]	1,514	579	2,093	(100%)
• Known vital status (May–June 2011)	1,469	579	2,048	(97.8%)
• Alive and living in Ozyorsk during the study	301	153	454	(21.7%)
• No. of exposed resident blood samples in 2011	18	82	**100**	**(4.8%)**
• No. of unexposed resident blood samples in 2011			**+ 50**	
			150	

Phase I (whole genome screening for radiation-associated genes) (*7*)

1. Random split sample set: phase I, 40[2] / phase II, 110
2. Screening set with 4 × 10 RNA samples comprising one unexposed (control) and three exposed groups[3]
3. Examining
   - transcriptional changes (whole genome | microarray, 19,596 genes)
     → ~500 mRNA (335 genes)[4]
     → gene enrichment analysis (PANTHER)
     → 95 mRNA for phase II
   - post-transcriptional changes (qRT-PCR, ~667 microRNA)
     → 45 microRNA[5] for phase II

Phase II (validation of radiation-associated genes on remaining samples) (*8*)

1. Independent validation set with 92 samples[6]
2. Employ six statistical models separately for each exposure type and gene[7]
3. Adjust significantly associated candidates (25 mRNA, 20 microRNA) with 26 confounders → 15 mRNA and 15 microRNA survived
4. Perform nonparametric Kruskal Wallis test (3 *df*) on the surviving candidate genes
5. Examine mRNA-microRNA relationship
6. Analytically determine association of final mRNA/microRNA candidates with blood cell counts
7. Laboratory technique validation on 40 samples used for the whole genome microarray (phase I) with qRT-PCR data from 92 samples (phase II)

Phase III (examining phase II genes and their association with chronic diseases)

1. Selection of chronic diseases and clinical parameters used for analysis[8] → 15 chronic diseases and 3 clinical parameters
2. Employing two statistical models, examine radiation dose–gene effect (chronic diseases/clinical parameters) association for 15 mRNA, 15 microRNA and 18 chronic diseases/clinical parameters[9]
3. Adjust models for co-linearity among diseases where needed[9]
4. Calculate odds ratio for models with persistent significant radiation-gene and effect-gene relationships[10]
5. Examine disease-dependent mRNA-microRNA relationships

[1]Total doses were >0.5 Gy for individuals solely exposed to external gamma ray. For individuals with combined occupational exposures total doses for external gamma ray exceeded 1.01 Gy and internal Pu body burden was >0.7 kBq.

[2]Samples for phase I were matched with age at exposure, age of biosampling and gender. Selected individuals received no radiotherapy and the gap between biosampling and death was >6 month. RIN of RNA was >7.5 and RNA quantity >7.5 μg.

[3]Controls served as reference (group 1). Exposed individuals were characterized by low gamma-ray exposure (mean: 0.7 Gy) and high Pu burden (mean: 0.09 kBq, group 2), high gamma-ray exposure (mean: 1.1 Gy) and low Pu burden (mean: 0.03 kBq, group 3) and high gamma-ray exposure (mean: 1.3 Gy) and no Pu burden (group 4).

[4]Search criteria: significant Kruskal Wallis *P* values among group comparisons and >twofold gene expression difference. Additionally we performed bioinformatic analysis for further reduction of the number of gene candidates.

[5]Normalized gene expression (normal distributed) served as outcome variable in generalized linear models containing four independent groups (control and three exposure groups). The reference was either the unexposed group or group 4 without Pu burden. We also employed separate linear models for the external gamma-ray exposure and the internal Pu burden.

[6]From the remaining 110 samples for phase II, 18 samples were lost due to malfunctioning consumable material leaving 92 samples eligible for phase II analysis.

[7]Normalized gene expression (normal distributed) served as outcome variable in generalized linear models. External gamma-ray exposure and internal Pu burden were categorized and separately used as explanatory variables in linear and categorical models. In addition, these models were adjusted for age at exposure and stratified by gender.

[8]Per disease at least 10 unexposed and 10 exposed (separately for both exposure types) male or female cases were required, thus 15 from 29 available chronic diseases eligible for further analysis were selected.

[9]In our first generalized linear model we employed normalized gene expression of our 15 mRNA and 15 microRNA candidates from phase II as outcome variable. Both external gamma-ray exposure and internal Pu burden were used as explanatory variables. Disease-dose associations were modeled by introducing an interaction term. In our second generalized linear model adjusted for both exposure types we examined the association of each of the 15 selected diseases with gene expression in separate models. Possible gene-dose associations interfering with the disease-gene association were controlled by adding an appropriate interaction term. Co-linearity of diseases was adjusted if required. Both models were stratified by gender.

[10]Logistic regression models were employed with the disease as the outcome variable and gene expression and exposures used as explanatory variable corresponding to those variables which contributed significantly in our second generalized linear model.

FIG. 1. Flow diagram depicting included samples, split study design, gene expression measurements and bioinformatics, with focus of current study on phase III. The top section on inclusion/exclusion criteria, Phase I/II refers to previous and recently published results on the same group (*7, 8*).

hypertension, ischemic heart disease, acute respiratory disease, blood diseases, infectious diseases, rheumatic diseases, skin diseases, tuberculosis, viral hepatitis, cerebrovascular disease, gastric ulcer, liver cirrhosis and connective tissue diseases. Due to the low frequencies of these 14 diseases we excluded them from analysis, leaving 15 diseases eligible for analysis, i.e., chronic radiation syndrome, benign tumors, diabetes mellitus, thyroid diseases, endocrine diseases, Parkinson's disease, atherosclerotic diseases, venous diseases, chronic pulmonary diseases, chronic gastritis, cholelithiasis, pancreas disease, kidney diseases, prostate hyperplasia and breast hyperplasia. These diseases partly comprise subgroups such as goiter, hypothyroidism and thyroiditis, which are classified as thyroid diseases following ICD-9 coding. Details are provided in Table 1 along with the corresponding ICD-9 codes. The study was approved by the Observation Council of the Southern Urals Biophysics Institute (Institutional Review Board, SUBI), which is responsible for implementation of ethical regulations. An informed consent to participate in the study was signed by each subject and approved by the Bundeswehr Institute of Radiobiology and SUBI.

TABLE 1
Disease Subgroup Summary

ICD-9	Disease subgroup	Unexposed[a]	Exposed[a]	ICD-9	Disease subgroup	Unexposed[a]	Exposed[a]
Benign tumors		**15**	**57**	**Atherosclerotic diseases**		**14**	**54**
210	Benign neoplasm of lip, oral cavity and pharynx	1	1	***440***	***Atherosclerosis***	***12***	***42***
211	***Benign neoplasm of other parts of digestive system***	***2***	***24***	441	Aortic aneurysm	1	5
212	Benign neoplasm of respiratory and intrathoracic organs	0	3	***443***	***Other peripheral vascular disease***	***2***	***22***
213	Benign neoplasm of bone and articular cartilage	1	1	**Venous diseases**		**19**	**63**
214	***Lipoma***	***3***	***16***	444	Arterial embolism and thrombosis	0	2
215	Other benign neoplasm of connective and other soft tissue	0	5	***451***	***Phlebitis and thrombophlebitis***	***3***	***13***
216	***Benign neoplasm of skin***	***5***	***17***	453	Other venous embolism and thrombosis	0	1
218	Uterine leiomyoma	6	8	***454***	***Varicose veins of lower extremities***	***15***	***48***
219	Other benign neoplasm of uterus	0	0	***455***	***Hemorrhoids***	***7***	***29***
222	Benign neoplasm of male genital organs	0	2	456	Varicose veins of other sites	1	2
223	Benign neoplasm of kidney and other urinary organs	0	2	**Chronic pulmonary diseases (491–494)**		**16**	**36**
224	Benign neoplasm of eye	0	1	**Chronic gastritis (535)**		**36**	**83**
227	Benign neoplasm of other endocrine glands and related structures	0	1	**Cholelithiasis (574, 575)**		**27**	**76**
228	Hemangioma and lymphangioma, any site	3	6	**Pancreas disease (577)**		**14**	**16**
229	Benign neoplasm of other and unspecified sites	0	1	**Kidney diseases**		**29**	**61**
238	Neoplasm of uncertain behavior of other and unspecified sites and tissues	0	1	580	Acute glomerulonephritis	0	1
Thyroid diseases		**12**	**61**	582	Chronic glomerulonephritis	0	3
240	***Simple and unspecified goiter***	***8***	***18***	583	Nephritis and nephropathy, not specified as acute or chronic	0	1
241	***Nontoxic nodular goiter***	***10***	***48***	584	Acute renal failure	1	0
242	Thyrotoxicosis with or without goiter	0	5	585	Chronic renal failure	0	5
244	***Acquired hypothyroidism***	***6***	***16***	587	Renal sclerosis, unspecified	1	0
245	***Thyroiditis***	***5***	***12***	***590***	***Infections of kidney***	***16***	***30***
246	Other disorders of thyroid	0	2	591	Hydronephrosis	1	0
Diabetes mellitus (250)		**7**	**20**	***592***	***Calculus of kidney and ureter***	***12***	***23***
Endocrine diseases		**30**	**59**	***593***	***Other disorders of kidney and ureter***	***14***	***40***
251	Other disorders of pancreatic internal secretion	0	1	**Prostatic hyperplasia (600)**		**8**	**47**
253	Disorders of the pituitary gland and its hypothalamic control	0	3	**Breast hyperplasia (610)**		**5**	**12**
256	Ovarian dysfunction	0	1	**Chronic radiation syndrome (CRS)**		**0**	**36**
272	***Disorders of lipoid metabolism***	***19***	***11***	**Body mass index (BMI) ($\leq$26 vs. $>$26)[b]**		**30**	**71**
274	***Gout***	***2***	***10***	**Systolic blood pressure ($\leq$140 vs. $>$140)[b]**		**18**	**46**
277	Other and unspecified disorders of metabolism	0	1	**Diastolic blood pressure ($\leq$80 vs. $>$80)[b]**		**16**	**27**
278	***Obesity and other hyperalimentation***	***20***	***49***	**Smoking (yes/no)[b]**		**6**	**45**
Parkinson's disease (332)		**5**	**25**	**Alcohol consumption (yes/no)[b]**		**18**	**76**

Note. Disease subgroups (coded according to the ICD-9 nomenclature) were summarized to diseases and are depicted herein.

[a] Shown are only the cases for the unexposed and exposed (both exposure types are summarized) individuals. Each disease and the total number of individuals with that disease are reflected in boldface. Disease subgroups and their frequency numbers are provided either in plain text (low frequency numbers) or boldface, italicized text (higher frequency numbers). The total numbers for the diseases and disease subgroups can differ since individuals may have one or a combination of subgroup diseases.

[b] Likewise, only the numbers of individuals with a more severe category disease (e.g., systolic blood pressure $>$140) are shown.

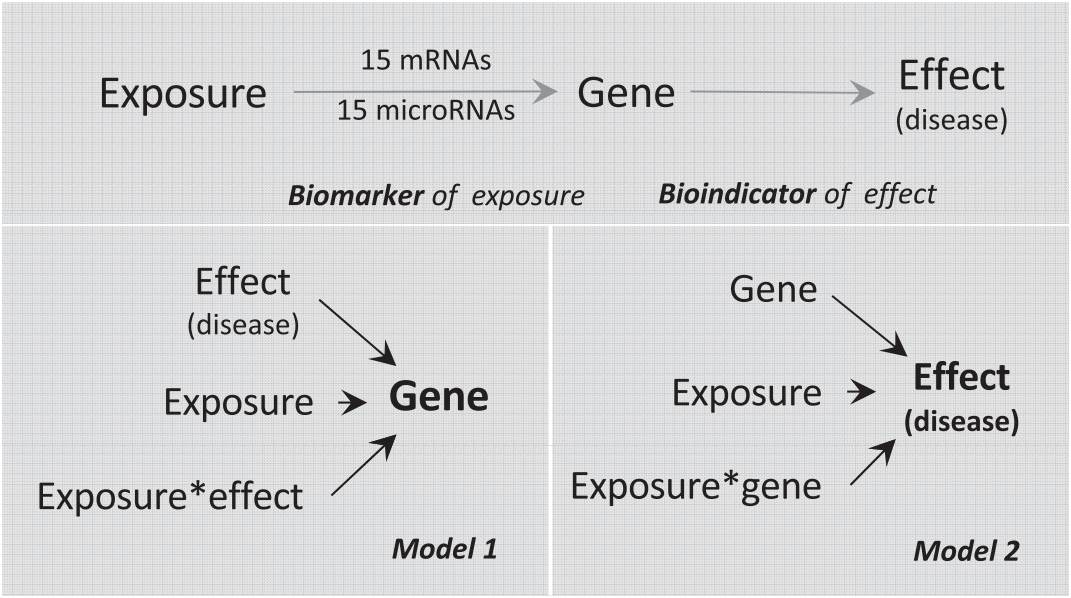

FIG. 2. In previous analysis we already identified significant exposure-to-gene relationships for 15 mRNAs and 15 microRNAs (biomarker of exposure, upper part). Following this causal pathway we wondered whether these genes might be also associated with a health effect, namely noncancerous chronic diseases (bioindicator of effect). To elucidate that we developed two models considering the relationships among the three components of our causal pathway as outlined in the lower part of the figure.

Dosimetry

Individual cumulative absorbed doses from external gamma rays and cumulative absorbed doses from external and internal radiation to red bone marrow (RBM) and other organs were estimated for every worker participating in the study. In particular, individual absorbed dose to the RBM were used for exposure analysis in this current study due to its significance as an appropriate exposure surrogate for the development of certain diseases. Estimates of plutonium exposure were based on the average of approximately nine urine sample measurements (*9–13*) and organ doses were calculated based on biokinetic models (*14–17*). Data on gamma-ray exposure from external sources were based primarily on film badge measurements (*18*). Further details can be found in ref. (*7*).

RNA Extraction and Quality Control

Whole blood samples (2.5 ml) were taken using the PAXgene™ Blood RNA system (PreAnalytiX GmbH; BD Diagnostics, Hombrechtikon, Switzerland) and processed accordingly [for details see ref. (*7*)].

Phase I Screening: Whole Genome Microarray and MicroRNA Experiments

Whole genome screening of 4 × 10 RNA samples was performed for differentially expressed genes (protein coding mRNAs) using the Agilent oligo microarray GE 8 × 60K (Agilent Technologies, Waldbronn, Germany). Four groups were constructed consisting of one unexposed group and 3 exposed groups, with the latter reflecting the strongest contrast of both occupational exposure types to the unexposed comparison subjects (Fig. 1). Exposed groups consisted of: 1. low gamma ray (mean, 0.7 Gy) and high plutonium exposure (mean, 0.09 Gy); 2. high gamma ray (mean, 1.1 Gy) and low plutonium exposure (mean, 0.03 Gy); and 3. high gamma ray (mean, 1.3 Gy) and negligible plutonium exposure (mean, 0.004 Gy). We used the nonparametric Kruskal-Wallis test (KW *P* value) to compare gene expression across three dose groups and the unexposed comparison subjects, and finally identified 95 gene candidates for validation by qRT-PCR in phase II [for details see ref. (*7*)] (Fig. 1).

We evaluated microRNA gene expression by qRT-PCR (TaqMan® primer probe assays) in the same 4 × 10 RNA samples using a low-density array (LDA) (type A&B; Life Technologies, Darmstadt, Germany). Altogether 667 microRNA could be analyzed simultaneously employing two 384-well LDAs, as described earlier [for details see ref. (*7*)] (Fig. 1). Altogether 45 microRNA candidates were identified for validation by qRT-PCR in phase II [for details see ref. (*7*)] (Fig. 1).

Phase II: Validation of Phase I Candidate Genes

For validation of the 95 candidate genes (mRNA) we employed a custom-made LDA and for validation of our 45 microRNA we used the same microRNA LDA (type A&B) as employed earlier in phase I, except that we restricted our analysis to the 45 microRNAs which appeared to be significantly associated with dose and we did not analyze the other 621 microRNA species. Hence, all data were generated employing different LDA qRT-PCR platforms. Altogether 15 mRNA and 15 microRNA species appeared to be significantly associated with the exposure types and were gender dependent (Fig. 1) while using linear models.

Phase III: Association of Radiation-Induced Genes with Diseases/ Health Status

Our purpose was to examine altered gene expression after radiation exposure as an intermediate along the causal pathway starting with two exposure types and leading to the occurrence of a chronic disease (Fig. 2, upper section). Exposure-to-gene, gene-to-disease as well as disease-exposure associations needed to be considered (Fig. 2, lower section). This required reconfirmation of the previously demonstrated exposure-to-gene association of our 15 mRNA and 15 microRNA species, while considering disease-exposure interactions as well. Therefore, after the first approach (examining exposure-to-gene associations), linear models were fitted to normalized gene expression

(GE, normally distributed) and its association with disease and the unexposed group as well as the three dose categories for internal plutonium (0–0.055 Gy; >0.055–0.085 Gy; >0.085) and external gamma-ray exposures (0–0.5 Gy; >0.5–1 Gy; >1 Gy) with cutoff points corresponding approximately to tertiles of dose distribution among the 92 cases from phase II. In addition, an interaction term of both disease and exposure was included to correct for potential synergistic influences on gene expression. The equation for the model can be written as:

$$\mathrm{GE} \sim \mathrm{exposure} + \mathrm{disease} + \mathrm{exposure}_{*}\mathrm{disease}.$$

All models were run separately for each disease, each exposure type and gene and were stratified by gender. If both exposure types contributed significantly to the model, we added them into one model. We calculated an exposure-to-gene 3 *df P* value (3 degrees of freedom *P* value, F test) over all four groups (3 exposed and 1 unexposed group), a 1 *df P* value for disease and a 1 *df P* value for disease*exposure interaction. If the interaction term did not contribute significantly to our models it was removed during further analysis. This was the case in almost all models.

After the second approach (examining gene-to-disease associations), logistic models were fitted to the probability of prevalent disease and its association with gene expression and exposure. Of note, for logistic regression analysis we regrouped exposed and unexposed individuals based on the occurrence (prevalent cases) or the absence of a disease outcome (non-cases). Again, an interaction term for potential synergistic influences of GE and exposure on disease was included. The equation for the model can be written as

$$\mathrm{Logit}(\mathrm{P_{dis}}) \sim \mathrm{GE} + \mathrm{exposure} + \mathrm{GE}_{*}\mathrm{exposure}.$$

where $\mathrm{P_{dis}}$ is the probability of disease and $\mathrm{Logit(P)} = \log[\mathrm{P}/(1–\mathrm{P})]$.

All models were stratified by gender and adjusted for the radiation exposure type of interest on a continuous or categorical scale, and the second exposure type was used on a continuous scale. We calculated Wald test *P* values for GE, exposure and the interaction term GE*exposure. Again, if the interaction term did not contribute significantly (which was observed in the vast majority of the models) it was removed during further analysis. Odd ratios (OR) reflecting increased risk for prevalent chronic diseases after altered gene expression were calculated for genes showing a significant exposure-to-gene association in the model 1, and a significant gene-to-disease association in model 2. We also calculated the concordance to quantify the discrimination ability among cases and non-cases based on gene expression measurements and radiation dose in these logistic regression models. All data analyses were done using SAS 9.2 (SAS Institute, Inc., Cary, NC).

Bioinformatic Analysis

Genes known to be associated with atherosclerosis (Unified Medical Language System®; http://www.nlm.nih.gov/research/umls/) were downloaded from the DisGeNET database (http://ibi.imim.es/web/DisGeNET/v01). Among them, HNRNPA1, RAPGEF1 and SERPINB9 were found to be associated with the gene-to-disease model, and were combined into a single list for uploading into the Reactome Pathway Database (http://www.reactome.org/). This list was mapped to the human interactome, as annotated in the Reactome Pathway Database using the Reactome functional interaction (FI) cytoscape plugin. Mapping was conducted allowing inclusion of so-called linker genes, i.e., genes from the interactome enabling indirect connections between genes from the input list.

RESULTS

Cohort Characteristics

As previously reported (*7*), unexposed and exposed groups (with the latter combined into one group due to low frequency of Mayak workers exposed to external gamma rays only) showed considerable differences regarding gender (unexposed: 70% females; exposed: 39% females). Several differences such as social behavior (unexposed group members smoke and drank less often), frequency differences in noncancer chronic diseases such as thyroid diseases (19% vs. 69% unexposed/exposed), atherosclerosis (26% vs. 62% unexposed/exposed) or diabetes (7% vs. 20%, unexposed/exposed) were caused by the female predominance in the group of unexposed individuals.

Mean cumulative red bone marrow dose from occupational external gamma-ray exposure (over all exposed groups) was 1.4 Gy (SD ± 0.51), ranging between 0.39 to 3.1 Gy. Mean cumulative absorbed red bone marrow dose from internal alpha-particle radiation due to plutonium uptake was 0.11 Gy (SD ± 0.13), ranging between 0.004 to 1.01 Gy. For calculating the equivalent dose these values have to be multiplied with an RBE of, for example, 5–20 to adjust for the end point-dependent radiobiological efficiency of alpha particles (*19*). Among Mayak PA workers we observed comparable ages of first exposure to either external gamma rays (median: 21; range: 17–31 years) or internal alpha particles of plutonium [median: 22; range: 17–35 years (*7*)].

Exposure-To-Gene (Model 1) and Gene-To-Disease (Model 2) Relationships

We examined the exposure-to-gene and the gene-to-disease relationships in 92 samples (47 males and 45 females) using the 15 mRNA and 15 microRNAs, which had already shown a significant and gender-dependent association with one or both exposure types (*7*). These examinations were performed in relationship to 15 chronic diseases and 3 clinical parameters of health status. For six different diseases, systolic blood pressure and BMI, we found up to 6 genes per disease that were significantly associated with both model 1 (exposure) and model 2 (disease) (Table 3). We expected significant gene-to-disease relationships in both models to be true for all genes (Table 3). However, gene-to-disease relationships in model 1, in particular for atherosclerosis-associated genes appeared to be insignificant due to the strong exposure-to-gene associations, but they became significant when employing exposure on a linear scale or removing the exposure variable (Table 3). Odds ratios typically ranged between 2.7 and 4.9 (interquartile range) and corresponding 95% CI appeared to be large (covering one log-scale) although significant. Concordance ranged between 71 and 81% (interquartile range, Table 3).

With an additional sensitivity analysis, we excluded the unexposed individuals from the models 1 and 2 analyses, leaving only exposed individuals eligible for the analysis. Corresponding results (**P* values) are marked with an asterisk (Table 3). The 2 *df* exposure* *P* values related to

TABLE 2
Disease Frequency and Clinical Parameters

Disease/clinical parameter	Categories	Unexposed		Exposed		
		n (50)	%	n (100)	%	χ^2
Benign tumors (210–238)	never	35	70	43	43	
	ever	15	30	57	57	0.002
Thyroid diseases (240–246)	never	38	76	39	39	
	ever	12	24	61	61	<0.0001
Diabetes mellitus (250)	never	43	86	80	80	
	ever	7	14	20	20	0.4
Endocrine diseases (251–278)	never	20	40	41	41	
	ever	30	60	59	59	0.9
Parkinson's disease (332)	never	45	90	75	75	
	ever	5	10	25	25	0.03
Atherosclerotic diseases (440–443)	never	36	72	46	46	
	ever	14	28	54	54	0.003
Venous diseases (444–456)	never	31	62	37	37	
	ever	19	38	63	63	0.004
Chronic pulmonary diseases (491–494)	never	34	68	64	64	
	ever	16	32	36	36	0.6
Chronic gastritis (535)	never	14	28	17	17	
	ever	36	72	83	83	0.1
Cholelithiasis (574, 575)	never	23	46	24	24	
	ever	27	54	76	76	0.006
Pancreas disease (577)	never	36	72	84	84	
	ever	14	28	16	16	0.08
Kidney diseases (580–593)	never	21	42	39	39	
	ever	29	58	61	61	0.7
Prostatic hyperplasia (600)	never	42	84	53	53	
	ever	8	16	47	47	0.0002
Breast hyperplasia (610)	never	45	90	88	88	
	ever	5	10	12	12	0.7
Chronic radiation syndrome	never	50	100	64	64	
	ever	0	0	36	36	<0.0001
Body mass index	≤26	20	40	29	29	
	>26	30	60	71	71	0.2
Systolic blood pressure	≤140	32	64	54	54	
	>140	18	36	46	46	0.2
Diastolic blood pressure	≤80	34	68	73	73	
	>80	16	32	27	27	0.5
Smoking	never	41	82	55	55	
	ever	6	12	45	45	0.0001
	missing	3				
Alcohol consumption	never	29	58	24	24	
	ever	18	36	76	76	<0.0001
	missing	3				

Notes. Frequency of diseases and clinical parameters are shown for unexposed (comparison subjects) and exposed (both exposure types combined) individuals of the whole group examined. Diseases and their subgroups are ordered according to their ICD-9 code (given in parentheses).

the exposure-to-gene and the 1 *df* disease* *P* values related to the exposure-to-diseases association among the exposed individuals in model 1 are shown on the left side of Table 3. For 34 exposure-to-gene associations, 10 became insignificant and 4 became borderline significant when excluding the unexposed individuals from the analysis (2 *df* exposure*); only 3 of 34 associations related to diseases appeared to be insignificant and 3 became borderline significant using model 1 (1 *df* disease*; Table 3). Likewise, 2 of 34 gene-to-disease associations (1 *df* gene*) in model 2 became insignificant and 4 became borderline significant after exclusion of unexposed individuals (Table 3, right side) leaving most of our associations for the exposed individuals significant.

Association of Identified Genes with the Atherosclerosis Interactome

Three of the radiation-associated genes (HNRNPA1, RAPGEF1 and SERPINB9) appeared to be related with atherosclerosis (Table 3), and were also on the list of genes known to be associated with atherosclerosis (DisGeNET database). We combined known genes from this database with the above-mentioned atherosclerosis-associated genes, and mapped this combination on the human

TABLE 3
Exposure-to-Gene and Gene-to-Disease Associations

			Exposure-to-gene association (first model)								
				Exposure categories							
Gene ID	Exposure	Gender	Total n	0 n	I n	II n	III n	**3 *df* Exposure**	2 *df* Exposure*	1 *df* Disease	1 *df* Disease*
Chronic radiation syndrome											
miR339-3p	External γ ray	Male	47	7	8	14	18	**0.003**	**0.005**	**0.03**	**0.03**
MCF2L	External γ ray	Male	45	7	7	14	17	**0.03**	0.06	**0.04**	**0.05**
miR151-3p	Internal Pu	Female	43	18	4	11	10	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
RAPGEF1	Internal Pu	Male	45	10	7	13	15	**0.006**	**0.02**	**0.04**[a]	**0.04****
Thyroid											
ATF6B	Internal Pu	Male	45	10	7	13	15	**0.0002**	0.2	**0.0006**	**0.001**
MGAT5	Internal Pu	Male	40	5	7	11	17	**0.001**	**0.02**	**0.001**	**0.02**
miR151-3p	External γ ray	Male	46	7	8	13	18	**0.006**	0.1	**0.03**	**0.04**
Systolic blood pressure (<140 vs. >140 mmHg)											
LOC731275	Internal Pu	Male	46	10	7	14	15	**0.002**	0.5	**0.01**	**0.02**
SGSM2	Internal Pu	Male	46	10	7	14	15	**0.0007**	0.1	**0.002**	**0.007**
SLC39A7	Internal Pu	Male	46	10	7	14	15	**0.0007**	0.06	**0.0009**	**0.001**
ZC3H7B	Internal Pu	Female	28	9	3	10	6	**0.007**	**0.009**	**<0.0001**	**0.002**
HNRNPA1	Internal Pu	Male	46	10	7	14	15	**0.0009**	**0.02**	**0.006**	**0.006**
HNRNPA1	External γ ray	Male	46	7	7	14	18	**0.01**	0.1	**0.006**	**0.009**
miR151-3p	Internal Pu	Female	43	18	4	11	10	**0.03**	**0.05**	**0.02**	**0.002**
Atherosclerosis (yes/no)											
AGAP4,6,8	Internal Pu	Male	45	10	7	13	15	**0.04**	**0.01**	**0.004****	**0.04**
RAPGEF1	Internal Pu	Male	45	10	7	13	15	**0.006**	**0.02**	**0.01****	**0.01**
SERPINB9	Internal Pu	Male	45	10	7	13	15	**0.0009**	**0.009**	**0.03****	**0.03****
HNRNPA1	Internal Pu	Male	46	10	7	14	15	**0.003**	**0.05**	**0.02****	**0.02****
Kidney diseases (yes/no)											
RNF166	Internal Pu	Male	45	10	7	13	15	**0.05**	0.4	0.05	0.07
miR484	Internal Pu	Female	44	19	4	11	10	**0.0005**	0.06	**0.03**	**0.02**
miR92a	Internal Pu	Female	44	19	4	11	10	**0.004**	**0.03**	**0.05**	0.09
miR151-3p	External γ ray	Male	46	7	8	13	18	**0.05**	0.4	**0.01**	**0.02**
miR501-5p	Internal Pu	Female	44	19	4	11	10	**0.02**	**0.007**	**0.03**	**0.02**
RNU48	Internal Pu	Female	44	19	4	11	10	**0.007**	**0.02**	**<0.0001**	**0.01**
Cholelithiasis (yes/no)											
RNF166	Internal Pu	Male	45	10	7	13	15	**0.003**	0.2	**0.02**	**0.02**
SGSM2	Internal Pu	Male	46	10	7	14	15	**0.0009**	0.4	**0.009**	**0.01**
HNRNPA1	Internal Pu	Male	46	10	7	14	15	**0.007**	**0.02**	**0.02**	**0.003**
Endocrine diseases (yes/no)											
miR92a	Internal Pu	Female	44	19	4	11	10	**0.0002**	**0.003**	**0.03**	**0.03**
miR451	Internal Pu	Female	44	19	4	11	10	**0.003**	0.06	**0.03**	0.4
miR19b	Internal Pu	Female	44	19	4	11	10	**0.01**	**0.04**	**0.05**	0.06
miR342-5p	Internal Pu	Female	42	18	4	11	9	**0.03**	0.1	**0.003**	0.7
RNU48	Internal Pu	Female	44	19	4	11	10	**0.001**	**0.02**	**0.02**	0.3
Benign tumors (yes/no)											
AGAP4,6,8	Internal Pu	Male	45	10	7	13	15	**0.03**	**0.04**	**0.02**	**0.01**
BMI (≤26 vs. >26)											
miR484	Internal Pu	Female	44	19	4	11	10	**<0.0001**	**0.02**	**0.01**	**0.05**

Notes. In a first linear model we compared normalized gene expression across the unexposed group and three dose categories for internal plutonium (Pu) (>0–0.055 Gy I, >0.055–0.085 Gy II, >0.085 Gy III) and external gamma-ray exposure (>0–0.5 Gy I, >0.5–1 Gy II, >1 Gy III). In addition, an interaction term disease-exposure was included to correct for potentially synergistic influences. If the interaction term did not contribute significantly to the models it was deleted during further analysis. All of these models were run separately for each disease and gene and stratified by gender. We calculated an exposure-to-gene 3 *df* F test *P* value over all four groups (three exposed groups and one unexposed group), a disease 1 *df P* value (**P* values indicate models employing exposure on a linear scale or removing the exposure variable) and a 1 *df P* value for the disease-exposure interaction term (not shown). In a second logistic model we employed disease as outcome variable, examined the relationship with normalized gene expression exposure and included an interaction term gene-exposure as well. It is important to note that for the logistic regression analysis we regrouped our exposed and unexposed individuals based on the occurrence (cases) or the absence of a disease (non-cases), which takes place in the exposed as well as the unexposed individuals. All models were stratified by gender and adjusted by the exposure type of interest on a linear scale. We calculated Wald test *P* values for gene, exposure and gene-exposure interaction term (not shown). With an additional sensitivity analysis we excluded the unexposed individuals from our model 1 and model 2 analyses leaving only exposed individuals eligible for analysis. Results (**P* values) are marked with an asterisk. The table includes the 2 *df* exposure* *P* values related to exposure and the 1 *df* disease* *P* values related to the diseases in the model 1 (left side of the table) and corresponding additional 1 *df* gene* *P* values restricted to exposed individuals only in model 2 (right side of the table). Significant associations are shown in boldface.

TABLE 3
Extended.

Gene-to-disease association (second model)										
Number Of non-cases	Number Of cases	1 *df* Exposure	**1 *df* Gene**	1 *df* Gene*	OR	95% CI		*P*	*P**	Concordance
33	14	0.02	**0.02**	**0.04**	4.2	1.1	15.9	**0.03**	0.05	77.3
31	14	0.01	**0.04**	0.06	0.4	0.1	1.01	0.05	0.08	78.8
27	16	0.2	**<0.0001**	**<0.0001**	3.1	1.5	6.3	**0.002**	**nd**	99.3
31	14	0.8	**0.05**	**0.05**	0.2	0.04	1.1	0.06	0.3	69.8
24	21	0.06	**0.02**	**0.0005**	5.1	1.039	24.9	**0.04**	**0.01**	71.0
22	18	0.005	0.06	**0.009**	3.5	0.898	13.6	0.07	**0.03**	78.0
24	22	0.0009	**0.04**	**0.04**	1.2	0.99	1.6	0.06	**0.05**	75.2
26	20	0.1	**0.02**	**0.02**	4.3	1.2	16.1	**0.03**	**0.04**	69.6
26	20	0.2	**0.009**	**0.008**	4.2	1.3	13.4	**0.02**	**0.02**	75
26	20	0.1	**0.003**	**0.003**	11.0	1.7	69.9	**0.01**	**0.02**	85.4
12	16	0.5	**0.001**	**0.003**	0.04	0.004	0.4	**0.007**	0.06	87.5
26	20	0.3	**0.02**	**0.02**	3.5	1.095	11.1	**0.03**	**0.05**	72.1
26	20	0.2	**0.01**	**0.02**	3.8	1.172	12.2	**0.03**	**0.04**	71.7
22	21	0.7	**0.01**	**0.002**	0.7	0.5	0.98	**0.04**	0.07	88.0
18	27	0.07	**0.005**	**0.04**	0.2	0.03	0.7	**0.01**	0.07	75.3
18	27	0.1	**0.02**	**0.1**	0.1	0.02	0.8	**0.03**	**0.04****	72.2
18	27	0.1	**0.02**	**0.05****	0.4	0.14	0.9	**0.04**	0.06**	70.4
18	28	0.1	**0.02**	**0.03****	0.3	0.093	0.9	**0.04**	**0.05****	73.8
17	28	0.9	**0.03**	**0.02****	0.2	0.1	1.0	**0.04**	**0.04****	69.3
13	31	nd	**0.0009**	**0.008**	3.4	1.4	8.5	**0.007**	**0.04**	76.9
13	31	nd	**0.004**	**0.04**	4.6	1.3	15.9	**0.02**	**0.02**	75.4
19	27	nd	**0.007**	**0.01**	0.7	0.6	1.0	**0.02**	**0.03**	68.8
13	31	nd	**0.001**	**0.003**	7.2	1.6	31.9	**0.009**	**0.03**	78.9
13	31	nd	**<0.0001**	**0.03**	8.6	2.1	34.1	**0.002**	**0.08**	85.4
17	28	0.09	**0.02**	**0.02**	0.2	0.04	0.9	**0.04**	**0.05**	75.0
17	29	0.2	**0.03**	**0.02**	0.3	0.1	0.97	**0.04**	**0.04**	74.2
17	29	0.3	**0.005**	**0.005**	0.2	0.057	0.7	**0.01**	**0.02**	74.2
15	29	0.04	**0.05**	0.06	2.5	0.9	7.2	0.08	0.2	67.4
15	29	0.02	**0.008**	0.09	2.7	1.1	6.4	**0.03**	0.2	75.9
15	29	0.05	**0.04**	**0.05**	2.0	0.9	4.3	0.07	0.1	69.7
15	27	0.06	**0.002**	0.3	4.9	1.5	15.5	**0.007**	**0.01****	79.3
15	29	0.03	**0.03**	0.9	2.6	1.04	6.5	**0.04**	0.9	70.6
21	24	0.02	**0.006**	**0.01**	0.16	0.04	0.7	**0.02**	**0.03**	78.6
10	34	0.02	**0.006**	0.07	3.7	1.2	11.3	**0.02**	0.1	80.6

interactome from the Reactome Pathway Database. From 452 genes uploaded, 231 genes including HNRNPA1, RAPGEF1 and SERPINB9 could be mapped to the interactome. The resulting partly directed network consisted of 272 nodes (231 atherosclerosis genes plus 41 linker genes) and 1,276 edges (interactions). We investigated the potential influence of each of the three atherosclerosis-associated genes separately as well as combined, by determining the first and second neighbors of these genes and subsequently calculated the percentage of their edges in the whole network. The first/second neighbors of HNRNPA1, RAPGEF1 and SERPINB9 alone covered 22–29% of the nodes and 70–77% of the edges of the atherosclerosis-associated interactome, respectively. All three genes combined covered 48% of all nodes and 93% of all edges of the atherosclerosis interactome. The first neighbors for HNRNPA1 were EP300 (E1A binding protein p300), ELAVL1 (ELAV like RNA binding protein 1), PARP1 [poly (ADP-ribose) polymerase 1] and NFIC [nuclear factor I/C (CCAAT-binding transcription factor)]. First neighbors for RAPGEF1 comprised PDGFB (platelet-derived growth factor beta), CDH1 (cadherin 1), PIK3R1 [phosphoinositide-3-kinase, regulatory subunit 1 (alpha)] and SRC [v-src avian sarcoma (Schmidt-Ruppin

A-2) viral oncogene homolog], while EGR1 (early growth response 1) and ESR1 (estrogen receptor 1) represented the first neighbors of SERPINB9.

DISCUSSION

In previous work we screened the whole genome and large parts of the miRNA transcriptome to search for exposure-to-gene relationships in 40 samples and then we validated those candidate genes by analyzing the remaining 92 samples with qRT-PCR (*7*). We found significant dose-to-gene associations in 15 mRNA and 15 microRNAs (*8*). We then considered whether these gene expressions, altered after radiation exposure, might be associated with increased risks of chronic diseases other than cancer and used two different models to analyze the exposure-to-gene (model 1) and the gene-to-disease associations (model 2), while considering all three components of this causal pathway in both models. These models were stratified by gender to allow for separate analysis of males and females, due to the gender dependency of gene expression values as already shown in previous analysis (*8*). We found significant exposure-to-gene and gene-to-exposure associations in both models for thyroid disease, endocrine diseases, benign tumors, BMI, chronic radiation syndrome, kidney diseases, cholelithiasis and systolic blood pressure. Odds ratios ranging between 2.7 and 4.9 (interquartile range) appeared to be high, which was certainly influenced by the low number of cases and non-cases, but might be also caused by the closer proximity of altered gene expression towards the effect (disease) along the causal pathway. For thyroid diseases and chronic radiation syndrome we would have expected exposure-to-gene associations, due to the known effect of radiation exposures on these diseases (*1, 20*). It is worth noting that gene expression changes of ATF6B are in agreement with previous work on another radiation exposed cohort, namely Chernobyl thyroid cancer patients after Iodine-131 exposure (*21, 22*) (Supplementary Table S1; http://dx.doi.org/10.1667/RR13758.1.S1). We also confirmed associations previously reported by others between SERPINB9, miR-342-5p and atherosclerosis, as well as between miR-151-3p and kidney disease (Supplementary Table S1).

Interestingly, incidence and mortality of cerebrovascular diseases (CeVD), mainly caused by atherosclerosis, appeared to be increased after prolonged exposure to low levels of radiation according to previous radio-epidemiological studies of the same cohort (*23, 24*). Similar findings were reported for the Life Span Study of Japanese A-bomb survivors (*25, 26*) after single-dose exposures as well as after fractionated radiotherapy with much higher doses (*27*). Recently, the National Council on Radiation Protection and Measurements (NCRP) emphasized the need for investigating genetic contributions of radiotherapy-associated CeVD (*27*). Genes such as PDGF (*28*) and certain cytokines and growth factors such as TGF-beta-1 and IL-2beta (*29*) may be involved. This is very much in line with our bioinformatic examinations, since PDGF represents one of the first neighbors of the radiation-associated candidate gene RAPGEF1. Other first neighbors of the three candidate genes reported here are also heavily involved in atherosclerosis, for example, through formation and destabilization of atherosclerotic plaques mediated by PARP1 (*30*), LDL retention in vascular walls involving the ROS → PKC, Src → caveolar or SNARE pathway (*31*), impact on the LDL cholesterol level in women (*32*) or by mediating endothelial cell inflammation through phosphorylation of transcriptional co-activator p300 (*33*). Another recent publication observed an insulin-like growth factor binding protein 5 (IGFBP5) mediated premature senescence in endothelial cells (HUVEC) after chronic (lasting several weeks) low-dose-gamma irradiation with 1.4 and 4.1 mGy/h (*34*). Radiation-induced premature senescence of endothelial cells is suggestive of a radiation-related increased risk of CeVD (*34*). Interestingly, a close member of the insulin-like growth factor family, IGFBP3, represents another second neighbor of our radiation-associated genes. In addition, our analysis identifies three radiation-associated genes (SERPINB9, HNRNPA1, RAPGEF1), and indicates that up to 93% of the whole atherosclerosis Reactome becomes altered through these three genes, an effect taking place even after chronic exposure to low levels of radiation.

To our knowledge, this is the first and largest study combining molecular biology with epidemiological data. We systematically screened the whole mRNA genome and large parts of the microRNA transcriptome for radiation-associated genes, and examined whether these altered genes might be associated with a health risk related to noncancer chronic diseases. Based on our preliminary data, chronic low-level radiation exposure seems to alter gene expression, which in turn causes increased health risk of noncancer chronic diseases. However, this study will need to be validated prospectively utilizing a larger group, and if possible, another exposed cohort.

There are several limitations to keep in mind when interpreting the results of this study. Since this analysis is exploratory, there are limitations due to low sample size and reduced power. Although with 92 individuals examined, this represents one of the largest and most complete *in vivo* gene expression studies of radiation-exposed persons, it remains problematic since the numbers were initially small and decreased further after stratification by gender, thus reducing the power of our analyses. This is also reflected by the large 95% CI sometimes covering one log-scale. Likewise, the satisfying concordances (0.71–0.81) should be interpreted cautiously, due to the small frequency of cases and non-cases.

Gene-to-disease relationships were examined based on exposure-to-gene associations using dose estimates to the RBM. Most associations were related to internal plutonium contamination. Given the close anatomical proximity of the cardiovascular system to the RBM or the immunological component of the chronic radiation sickness (CRS), dose estimates to the RBM appear to represent a reasonable exposure surrogate for effects observed in the vascular system and the CRS of our cohort. Dose estimates of

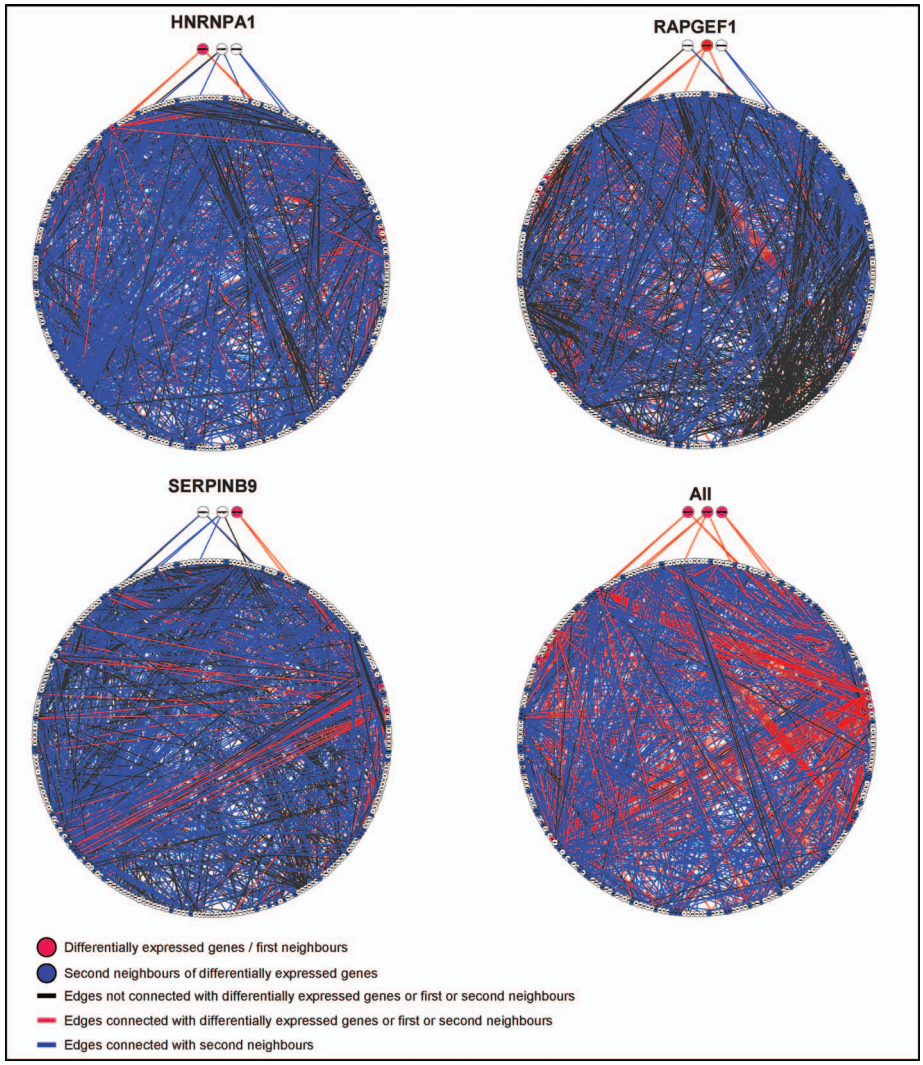

FIG. 3. Genes known to be associated with atherosclerosis (DisGeNET database) including our three atherosclerosis-associated genes (HNRNPA1, RAPGEF1, SERPINB9) were mapped on the human interactome from the Reactome Pathway Database. The resulting network consists of 272 nodes (genes, represented as circles) and 1,276 edges (interactions, represented as lines). We investigated the potential influence of each of the three atherosclerosis-associated genes on the atherosclerosis interactome separately and combined.

internal plutonium to liver, kidney and gastrointestinal tract were highly correlated with RBM dose estimates (Pearson correlation coefficient >0.99, $P < 0.0001$ and explained variance with $r^2 > 0.99$, $P < 0.0001$ examined in univariate linear-regression models), indicating that the RBM dose estimate represents a meaningful exposure surrogate for diseases such as cholelithiasis, kidney and gastrointestinal-related endocrine diseases (Table 3).

Furthermore, our gene expression was measured in blood samples of individuals already diagnosed with noncancer chronic diseases. This presents questions about the causal gene-to-disease relationship, since altered gene expression should technically be detected before onset of the disease. Based on the study design and in particular the time of blood draw we cannot differentiate whether the gene expression changes measured are modulated by the disease. However, analytically, we revealed significant dose-to-gene associations in model 1, which were adjusted for the impact of the disease-to-gene association as well (Fig. 2). The dose-to-gene associations remained significant even after these adjustments (Table 3). Following the bioinformatic approach, we demonstrated the impact of three radiation-responsive genes on the atherosclerotom (Reactome Pathway Database; Fig. 3). Finally, our further analysis using model 2 showed significant gene-to-disease relationships with most of the diseases related to the atherosclerotom. We interpret this as two independent indications for the impact of certain radiation-responsive genes on the atherosclerotom and associated diseases. Furthermore, our studies of Mayak PA workers as well as Chernobyl cohorts indicate that radiation exposure induces persistent gene expression changes through, for example, epigenetic modifications, interchromosomal aberrations or complex chromosome aberrations, as discussed above. Nevertheless, examinations

of biosamples collected before the occurrence of noncancer chronic diseases would be preferable, and this approach will be included in future plans to study another cohort.

One potential concern is whether our findings reflect surveillance bias. However, this would only be more likely if greater effort were put towards detecting diseases among workers with the highest radiation exposures. In this current study medical follow-up for every member of the cohort (including unexposed individuals) was conducted on a regular basis according to a specially developed standardized procedure, ensuring that all workers were examined uniformly irrespective of their exposures.

Another potential concern is related to the unexposed individuals in our analysis who worked as administrative workers in close proximity to the Mayak production side, and who underwent the same medical surveillance in the same hospital of the restricted area. Since they differed from the exposed individuals this added an uncontrolled aspect to our study design, and therefore there was concern that this might bias our results. With an additional sensitivity analysis, we excluded the unexposed individuals from models 1 and 2. This left only exposed individuals, who either did or did not report prevalent disease at the time of blood collection, as eligible for inclusion. Most of our associations remained significant even after restriction to the exposed individuals only (Table 3). This is consistent with a previous analysis of the Mayak cohort where we had already examined exposure-to-gene associations of our 15 mRNA and 15 microRNAs, and where unexposed individuals were excluded [2df *P* values at Table 2; see (*8*)].

In summary, radiation exposure appears to alter the expression of certain genes, which might increase the risk for the occurrence of noncancer chronic diseases, and particularly those related to atherosclerotic processes.

SUPPLEMENTARY INFORMATION

Table S1. Annotation of genes (12 mRNAs and 9 microRNAs) with noncancer chronic diseases and radiation exposure.

ACKNOWLEDGMENTS

This work was supported by the German Ministry of Defense. We appreciate the technical assistance provided by Mrs. Taubert in isolation of total RNA and the editorial support provided by Dr. Moroni from the Armed Forces Radiobiology Research Institute, Bethesda, MD. We are also deeply grateful for the substantial improvements and very constructive suggestions of the unknown associate editor.

Received: April 11, 2014; accepted: December 2, 2014; published online: February 23, 2015

REFERENCES

1. Sources and effects of ionizing radiation. United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) 2000 Report to the General Assembly, with scientific annexes. New York: United Nations, UNSCEAR; 2000.
2. Hande MP, Azizova TV, Burak LE, Khokhryakov VF, Geard CR, Brenner DJ. Complex chromosome aberrations persist in individuals many years after occupational exposure to densely ionizing radiation: an mFISH study. Genes Chromosomes Cancer 2005; 44:1–9.
3. Hande MP, Azizova TV, Geard CR, Burak LE, Mitchell CR, Khokhryakov VF, et al. Past exposure to densely ionizing radiation leaves a unique permanent signature in the genome. Am J Hum Genet 2003; 72:1162–70.
4. Mitchell CR, Azizova TV, Hande MP, Burak LE, Tsakok JM, Khokhryakov VF, et al. Stable intra-chromosomal biomarkers of past exposure to densely ionizing radiation in several chromosomes of exposed individuals. Radiat Res 2004; 162:257–63.
5. Anspaugh LR, Degteva MO, Vasilenko EK. Mayak Production Association: Introduction. Radiat Environ Biophys 2002; 41:19–22.
6. Azizova TV, Day RD, Wald N, Muirhead CR, O'Hagan JA, Sumina MV, et al. The ''Clinic'' medical-dosimetric database of Mayak production association workers: structure, characteristics and prospects of utilization. Health Phys 2008; 94:449–58.
7. Abend M, Azizova T, Müller K, Dörr H, Senf S, Kreppel H, et al. Gene expression analysis in Mayak workers with occupational prolonged radiation exposure. Health Phys 2014; 106: 664–76.
8. Abend M, Azizova T, Müller K, Dörr H, Senf S, Kreppel H, et al. Independent validation of candidate genes identified after a whole genome screening on Mayak workers exposed to occupational prolonged radiation exposure. Radiat Res 2014; 182:299–309.
9. Khokhryakov VF, Kudravtseva TI, Chernikov VI, Suslova KG, Orlova IA, et al. A scintillation method for determination of actinide alpha-activity in samples. J Radioanal Nucl Chem 1998; 234:293–95.
10. Krahenbuhl MP, Slaughter DM, Wilde JL, Bess JD, Miller SC, Khokhryakov VF, et al. The historical and current application of the FIB-1 model to assess organ dose from plutonium intakes in Mayak workers. Health Phys 2002; 82:445–54.
11. Fountos BN, Rabovsky JL. The Department of Energy's Russian Health Studies Program. Health Phys 2007; 93:187–89.
12. Vasilenko EK, Khokhryakov VF, Miller SC, Fix JJ, Eckerman K, Choe DO, et al. Mayak worker dosimetry study: an overview. Health Phys 2007; 93:190–206.
13. Vasilenko EK, Scherpelz RI, Gorelov MV, Strom DJ, Smetanin MY. External dosimetry reconstruction for Mayak workers. Presented at the AAHP Special Session Health Physics Society Annual Meeting. Salt Lake City; 2010. (http://bit.ly/1BJ03kF)
14. Khokhryakov VF, Menshikh ZS, Suslova KG, Kudryavtseva TI, Tokarskaya ZB, et al. Plutonium excretion model for the healthy man. Radiat Protec Dosim 1994; 53:235–9.
15. Khokhryakov V, Suslova K, Aladova E, Vasilenko E, Miller SC, Slaughter DM, et al. Development of an improved dosimetry system for the workers at the Mayak Production Association. Health Phys 2000; 79:72–6.
16. Khokhryakov VF, Suslova KG, Vostrotin VV, Romanov SA, Menshikh ZS, et al. The development of the plutonium lung clearance model for exposure estimation of the Mayak Production Association, nuclear plant workers. Health Phys 2002; 82:429–31.
17. Khokhryakov VV, Khokhryakov VF, Suslova KG, Vostrotin VV, Vvedensky VE, Sokolova AB, et al. Mayak Worker Dosimetry System 2008 (MWDS-2008): assessment of internal dose from measurement results of plutonium activity in urine. Health Phys 2013; 104:366–78.
18. Romanov SA, Vasilenko EK, Khokhryakov VF, Jacob P. Studies on the Mayak nuclear workers: dosimetry. Radiat Environ Biophys 2002; 41:23–8.
19. Hall E, Giaccia AJ. Radiobiology for the radiologist. 7th ed. Philadelphia: Lippincott Williams & Wilkins; 2012.
20. Shilnikova NS, Koshurnikova NA, Bolotnikova MG, Kabirova NR, Kreslov VV, Lyzlov AF, et al. Mortality among workers with chronic radiation sickness. Health Phys 1996; 71:86–9.
21. Abend M, Pfeiffer RM, Ruf C, Hatch M, Bogdanova TI, et al. Iodine-131 dose dependent gene expression in thyroid cancers and

corresponding normal tissues following the Chernobyl accident. PLoS One 2012; 7:e39103.

22. Abend M, Pfeiffer RM, Ruf C, Hatch M, Bogdanova TI, Tronko MD, et al. Iodine-131 dose-dependent gene expression: alterations in both normal and tumour thyroid tissues of post-Chernobyl thyroid cancers. Br J Cancer 2013; 109:2286–94.

23. Azizova TV, Muirhead CR, Druzhinina MB, Grigoryeva ES, Vlasenko EV, Sumina MV, et al. Cardiovascular diseases in the cohort of workers first employed at Mayak PA in 1948–1958. Radiat Res 2010; 174:155–68.

24. Azizova TV, Muirhead CR, Druzhinina MB, Grigoryeva ES, Vlasenko EV, Sumina MV, et al. Cerebrovascular diseases in the cohort of workers first employed at Mayak PA in 1948–1958. Radiat Res 2010; 174:851–64.

25. Preston DL, Shimizu Y, Pierce DA, Suyama A, Mabuchi K. Studies of mortality of atomic bomb survivors. Report 13: solid cancer and noncancer disease mortality: 1950–1997. Radiat Res 2003; 160:381–407.

26. Shimizu Y, Kodama K, Nishi N, Kasagi F, Suyama A, Soda M, et al. Radiation exposure and circulatory disease risk: Hiroshima and Nagasaki atomic bomb survivor data, 1950–2003. Br Med J 2010; 340:b5349.

27. Travis LB, Ng AK, Allan JM, Pui CH, Kennedy AR, Xu XG, et al. Second malignant neoplasms and cardiovascular disease following radiotherapy. Health Phys 2014; 106:229–46.

28. Li M, Jendrossek V, Belka C. The role of PDGF in radiation oncology. Radiat Oncol 2007; 11:2–5.

29. Basavaraju SR, Easterly CE. Pathophysiological effects of radiation on atherosclerosis development and progression, and the incidence of cardiovascular complications. Med Phys 2002; 29:2391–403.

30. Xu S, Bai P, Little PJ, Liu P. Poly(ADP-ribose) Polymerase 1 (PARP1) in atherosclerosis: from molecular mechanisms to therapeutic implications. Med Res Rev 2013; 34:644–75.

31. Bian F, Yang X, Zhou F, Wu PH, Xing S, Xu G, et al. CRP promotes atherosclerosis by increasing LDL transcytosis across endothelial cells. Br J Pharmacol 2014; 171:2671–84.

32. Jamshidi Y, Gooljar SB, Snieder H, Wang X, Ge D, Swaminathan R, et al. SHP-2 and PI3-kinase genes PTPN11 and PIK3R1 may influence serum apoB and LDL cholesterol levels in normal women. Atherosclerosis 2007; 194:e26–33.

33. Zhang Y, Qiu J, Wang X, Zhang Y, Xia M. AMP-activated protein kinase suppresses endothelial cell inflammation through phosphorylation of transcriptional coactivator p300. Arterioscler Thromb Vasc Biol 2011; 31:2897–908.

34. Rombouts C, Aerts A, Quintens R, Baselet B, El-Saghire H. Transcriptomic profiling suggests a role for IGFBP5 in premature senescence of endothelial cells after chronic low dose rate irradiation. Int J Radiat Biol 2014; 90:560–74.