Anaplastic lymphoma kinase (ALK) gene rearrangements in radiation-related human papillary thyroid carcinoma after the Chernobyl accident

Annette Arndt1, Konrad Steinestel1, Alexis Rump2, Manveer Sroya3, Tetiana Bogdanova4, Leonila Kovgan5, Matthias Port2, Michael Abend2 and Stefan Eder2,6*

1 Institute of Pathology and Molecular Pathology, Bundeswehrkrankenhaus Ulm, Ulm, Germany
2 Bundeswehr Institute of Radiobiology, Munich, Germany
3 Imperial College London, Charing Cross Hospital, London, UK
4 State Institution V.P. Kommissarenko Institute of Endocrinology and Metabolism of the National Academy of Medical Sciences of Ukraine, Kiev, Ukraine
5 Division of Dosimetry and Radiation Hygiene, Scientific Research Center for Radiation Medicine, Kiev, Ukraine
6 Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, Inner City Clinic, University Hospital of Munich (LMU), Munich, Germany

*Correspondence to: Stefan Eder, Bundeswehr Institute of Radiobiology, Neuherbergstrasse II, 80937 Munich, Germany. E-mail: stefanfriedricheder@bundeswehr.org

Abstract

Childhood radiation exposure has been associated with increased papillary thyroid carcinoma (PTC) risk. The role of anaplastic lymphoma kinase (ALK) gene rearrangements in radiation-related PTC remains unclear, but STRN-ALK fusions have recently been detected in PTCs from radiation exposed persons after Chernobyl using targeted next-generation sequencing and RNA-seq. We investigated ALK and RET gene rearrangements as well as known driver point mutations in PTC tumours from 77 radiation-exposed patients (mean age at surgery 22.4 years) and PTC tumours from 19 non-exposed individuals after the Chernobyl accident. ALK rearrangements were detected by fluorescence in situ hybridisation (FISH) and confirmed with immunohistochemistry (IHC); point mutations in the BRAF and RAS genes were detected by DNA pyrosequencing. Among the 77 tumours from exposed persons, we identified 7 ALK rearrangements and none in the unexposed group. When combining ALK and RET rearrangements, we found 24 in the exposed (31.2%) compared to two (10.5%) in the unexposed group. Odds ratios increased significantly in a dose-dependent manner up to 6.2 (95%CI: 1.1, 34.7; p = 0.039) at Iodine-131 thyroid doses >500 mGy. In total, 27 cases carried point mutations of BRAF or RAS genes, yet logistic regression analysis failed to identify significant dose association. To our knowledge we are the first to describe ALK rearrangements in post-Chernobyl PTC samples using routine methods such as FISH and IHC. Our findings further support the hypothesis that gene rearrangements, but not oncogenic driver mutations, are associated with ionising radiation-related tumour risk. IHC may represent an effective method for ALK-screening in PTCs with known radiation aetiology, which is of clinical value since oncogenic ALK activation might represent a valuable target for small molecule inhibitors.

Keywords: Chernobyl; papillary thyroid carcinoma; ionising radiation; ALK; RET; BRAF

Introduction

The risk of papillary thyroid carcinoma (PTC) has been clearly associated with a history of ionizing radiation exposure, especially at young ages [1]. The higher metabolic activity makes the gland especially vulnerable to the carcinogenic effects of radioactive Iodine-131 during childhood and adolescence. Follow-up after the Chernobyl nuclear accident in 1986 revealed a striking increase in the incidence of childhood PTC among the population in the highly I-131 contaminated areas of Belarus and Ukraine. A strong relation between I-131 thyroid dose and the risk of PTC has been shown [2,3]. Additionally,
long-term cohort studies substantiated the strong association between the risk of PTC and young age at exposure [2,4,5].

Investigations of the molecular mechanisms possibly underlying PTC revealed mutually exclusive genetic aberrations that cause constitutive activation along the MAPK-signalling pathway [6]. Yet, the molecular profile of PTC appears also to be strongly influenced by age at diagnosis [7]. While the prevalence of point mutations in the BRAF and RAS genes found in sporadic PTC cases rises simultaneously with increasing age at diagnosis, chromosomal rearrangements of the RET/PTC and TRK genes are more common in childhood and adolescent PTC cases than in adult onset PTC [7–13].

As radiation-induced DNA double strand breaks can cause chromosomal rearrangements, it was hypothesized that PTC cases diagnosed after radiation exposure could show a high prevalence of such fusion events. Indeed, some studies of post-Chernobyl PTC cases revealed an association of RET/PTC and NTRK rearrangements with individual I-131 thyroid doses, but up to one-third of radiation-related tumours harboured none of the known mutations [14]. Efforts have continued to discover other targets, resulting in the identification of new gene rearrangements such as PAX8/PPARγ, AKAP9/BRAF, or TPR/NTRK1 [14–18]. Recently, rearrangements of the anaplastic lymphoma kinase (ALK) gene were identified in PTCs of atomic bomb survivors and individuals exposed in the Chernobyl accident, suggesting a possible role of ALK rearrangements in radiation-related PTC [16,19].

ALK encodes for a membrane tyrosine kinase receptor, which is physiologically expressed in fetal neuronal progenitor cells and plays a key role in cell proliferation, survival, and differentiation [17]. Aberrant non-neuronal expression of ALK caused by rearrangement events has been shown to drive the carcinogenesis of various malignancies, including anaplastic large cell lymphoma, a subset of non-small-cell lung cancer (NSCLC) and, more recently, aggressive forms of thyroid cancer [15,17,18]. Targeted therapy with ALK inhibitors like Crizotinib is in clinical use for the treatment of ALK-rearranged NSCLC and offers a promising therapeutic option for other ALK-driven tumours [20]. The diagnosis of ALK rearrangements is required prior to ALK-inhibiting therapy and can be routinely performed using immunohistochemistry (IHC) or fluorescence in situ hybridisation (FISH).

In this study, we screened young Ukrainian PTC patients exposed to I-131 fallout after the Chernobyl nuclear accident for common driver mutations and gene rearrangements, including ALK, by DNA pyrosequencing, IHC and FISH to investigate whether a molecular radiation signature can be established using routine diagnostic techniques.

Materials and methods

DNA and tissue samples

We studied tissue samples from 100 Ukrainian patients who were diagnosed with PTC and underwent surgery between 1999 and 2013. Of these, 80 patients were exposed to I-131 fallout during the Chernobyl accident. Twenty were born after 31 March 1987 and, due to the isotope’s physical half-life of approximately 8 days, were considered unexposed. Pathological diagnoses were performed by the Institute of Endocrinology and Metabolism (Kiev, Ukraine) and were reviewed by the International Pathology Panel of the Chernobyl Tissue Bank (CTB). Preparation of tissue microarrays (TMA) by arranging formalin-fixed paraffin-embedded (FFPE) archive tissue samples, and isolation of corresponding tumour DNA, were carried out by the CTB (CTB project number 002/14). We obtained a total of nine TMA slides consisting of three different TMAs containing cores of varying areas of the respective tumour.

Sample inclusion and exclusion criteria

We excluded four samples from the study because tumour tissues from three persons were not present across all analysed TMA slides and, for one person, no thyroid dose estimate was available. Pyrosequencing analysis of point mutations in BRAF or RAS genes was performed on 95 samples, because an additional sample lacked a DNA sample. FISH analysis of RET gene rearrangements was performed using a single TMA slide and included a total of 69 samples due to missing cores or unrepresentative tissue. Results for IHC-testing are based on the analysis of 93 PTCs, representing the number of samples present on two TMA slides stained by IHC.

One sample could not be analysed by FISH as a consequence of high background fluorescence. This sample was included in the study and regarded as ALK-non-rearranged due to negative IHC staining.

I-131 thyroid dose estimates

Individual thyroid doses from I-131 fallout exposure of persons diagnosed with PTC were estimated by
ALK rearrangements in post-Chernobyl PTC samples

V. V. Vladi, S. N. Nikiforova, I. M. Vagner, Yu. V. Khromov, S. V. Svetlikov, A. G. Svetlikov, A. M. Makarov, and S. N. Nikiforova

the Ukrainian Institute of Radiation Protection (Kiev, Ukraine) as previously described [21,22]. In brief, for each CTB donor, 1000 stochastic dose estimates were calculated while randomly changing values of uncertain parameters and the resulting arithmetic mean was used as a single thyroid dose value for statistical analysis.

Pyrosequencing

Sequence analysis of mutational hot-spot regions in KRAS, NRAS, and BRAF oncogenes was performed using the PyroMark Q24 platform and the In Vitro Diagnostic marked therascreen KRAS, RAS extension, and BRAF pyro kits according to the instructions of the manufacturer (Qiagen, Hilden, Germany). In brief, the respective genetic regions of interest were amplified using biotin-labelled primers and 10 ng of patient DNA per PCR approach. After denaturation and purification steps, the single stranded PCR products were used as templates for sequence analysis.

FISH and IHC analyses

FISH analysis was performed on TMAs using the ZytoLight SPEC ALK/EML4 TriCheck™Probe or the ZytoLight SPEC RET Dual Colour Break Apart Probe. Pre-treatment, denaturation, hybridization as well as washing steps were performed as recommended by the manufacturer (Zytomed Systems, Berlin, Germany). For evaluation, at least 50 tumour cells were taken into account per core. Cases were considered positive when ≥15 cells showed break-apart signals.

ALK-IHC was also conducted on TMA slides applying the anti-human ALK monoclonal primary antibody clone 1A4 in a 1:100 dilution (Zytomed Systems). Heat-induced epitope retrieval was done in CC1-buffer for 72 min at 95 °C followed by incubation of anti-ALK antibody for 16 min at 37 °C. Subsequent detection was performed using the OptiView DAB IHC Detection Kit. All steps of the IHC assay were carried out on the Ventana Benchmark XT System (Ventana Medical Systems, Tucson, AZ, USA). Estimation of ALK expression was done by scoring the intensity of cytoplasmic staining in at least 10% of tumour cells assigning scores from 0 to 3+ [23].

Statistics

We examined an association of genetic mutation and rearrangement frequencies with different exposure categories among our case series. Descriptive statistics of continuous (n, mean, standard deviation, min, max) and categorical variables (frequency distributions) were performed and corresponding P values (t-test/non-parametric rank sum tests, as well as chi-square/Fisher’s exact test, where applicable) were calculated for each of the variables. Dose was categorised following this categorisation (0, >0–100, >100–500, >500 mGy) and a categorisation resulting in an even distribution (0, >0–60, >60–300, >300 mGy) of thyroid cancer samples among the dose strata. Frequency distributions of BRAF/RAS mutations and ALK and RET rearrangements separately and combined were examined for both types of dose categorization. Cumulative unconditional logistic regression analysis was performed on dose categories as outcome variable. Summed BRAF/RAS mutations and all rearrangements combined were used as explanatory variables in univariate analysis. To these models, we added potential confounders such as gender and age at exposure/surgery in order to adjust for their impact on the association of genetic alterations and dose categories. Odds ratios (ORs), 95% confidence intervals (95% CIs), and corresponding P values (Wald Chi-square) were calculated. Cox-proportional hazard regression models with tumour latency as the time scale was employed on the exposed group using the lowest dose group as the reference. Violation of the proportional hazard assumption was examined for each variable separately. We generated a time-dependent covariate by creating interactions of the predictors and a function of latency time and included them in the model. The model with combined ALK and RET rearrangements as the explanatory variable was finally adjusted for gender as a potential confounder using multivariate models.

All calculations were performed using SAS (release 9.2, Cary, NC, USA). Data were visualised using Sigma Plot (Version 13, Systat software GmbH, Erkrath, Germany).

Results

Characteristics of the PTC cases, exposure, and biological endpoints

The tumour samples were from 25 male (26%) and 71 female (74%) residents of Ukrainian regions (oblasts; Table 1). The unexposed group included 19 cases (19.8%) with a mean birth date of 5.2 years after the Chernobyl accident. All 77 exposed patients (80.2%) were younger than 18 years at the time of the accident (mean age = 5.5 years).
Most of our unexposed samples were from female patients (18 of 19, 94.7%) compared to 68.8% of female patients in the exposed group ($p = 0.02$; Table 1). About 80–95% of both groups originated either from the Kiev, Rovno, Sumy, or Zhytomyr oblast ($p = 0.1$) and showed a comparable age at surgery (21.3 ± 4.3 versus 22.4 ± 5.5 years in the unexposed versus the exposed group, respectively, Table 1). Surgery was performed with an average of 16.9 years after exposure ($p = 0.46$).

Individual Iodine-131-thyroid doses ranged from 13 mGy to a maximum value of 2560 mGy. The mean exposure dose was 359 mGy (±481 mGy; Table 1). We used a standardised categorisation (0, >0–100, >100–500, >500 mGy) and a categorization resulting in an even distribution (0, >0–60, >60–300, >300 mGy) of the exposed group members so that about 25–28% fell into one of the three dose categories (Table 1).

Among the 96 examined biopsies, we detected 7 $ALK$ rearrangements ($ALK^+$, break-apart or isolated red signal in FISH analysis) in the exposed group and none in the unexposed group (Figure 1 and Table 2). Detailed patient characteristics of the

### Table 1. Descriptive characteristics of the radiation unexposed and exposed groups diagnosed with PTC after the Chernobyl accident

| Characteristic | Category | Unexposed ($n = 19$) | | | Exposed ($n = 77$) | | | $P$ value |
|---------------|----------|----------------------|---|---|-------------------|---|---|---|
| Gender        | Female   | 18 94.7%             | | | 53 68.8%          | | | 0.02 |
|               | Male     | 1 5.3%               | | | 24 31.2%          | | |   |
| Oblast        | Chercassy| 0 0%                 | | | 3 3.9%            | | |   |
|               | Chernigov| 1 5.3%               | | | 11 14.3%          | | |   |
|               | Kiev     | 12 63.2%             | | | 27 35.1%          | | |   |
|               | Rovno    | 3 15.8%              | | | 8 10.4%           | | |   |
|               | Sumy     | 2 10.5%              | | | 7 9.1%            | | |   |
|               | Zhytomyr | 1 5.3%               | | | 21 27.3%          | | | 0.14 |
| Age at incidence | $n$ | 19 | | | 77 | | |   |
|               | Mean     | −5.2                 | | | 5.5               | | |   |
|               | SD       | 4.2                  | | | 5.1               | | |   |
|               | Min      | −17.5                | | | 0.1               | | |   |
|               | Max      | −0.9                 | | | 17.7              | | | NA  |
| Age at surgery | $n$ | 19 | | | 77 | | |   |
|               | Mean     | 21.3                 | | | 22.4              | | |   |
|               | SD       | 4.3                  | | | 5.5               | | |   |
|               | Min      | 9.4                  | | | 14                | | |   |
|               | Max      | 26.6                 | | | 33.7              | | | 0.46 |
| Time between exposure and surgery | $n$ | 19 | | | 77 | | |   |
|               | Mean     | NA                   | | | 16.9              | | |   |
|               | SD       | NA                   | | | 2.9               | | |   |
|               | Min      | NA                   | | | 12                | | |   |
|               | Max      | NA                   | | | 25.2              | | | NA  |
| Variable      | Category (mGy) | Exposed ($n = 77$) | | | | | | |
|               | all      | 77 100.0%            | | | 359.1             | | | 480.8 |
| Dose categories | >0–100 | 29 37.7%             | | | 41.2              | | | 19.0  |
|               | >100–500 | 29 37.7%             | | | 243.8             | | | 106.3 |
|               | >500     | 19 24.6%             | | | 1020.2            | | | 558.9 |
|               | >0–60    | 24 31.2%             | | | 34.0              | | | 10.2  |
|               | >60–300  | 27 35.1%             | | | 172.8             | | | 73.3  |
|               | >300     | 26 33.7%             | | | 852.5             | | | 552.2 |
|               | all      | 77 100.0%            | | | 359.1             | | | 480.8 |
|               | >0–100   | 29 37.7%             | | | 41.2              | | | 19.0  |
|               | >100–500 | 29 37.7%             | | | 243.8             | | | 106.3 |
|               | >500     | 19 24.6%             | | | 1020.2            | | | 558.9 |
|               | >0–60    | 24 31.2%             | | | 34.0              | | | 10.2  |
|               | >60–300  | 27 35.1%             | | | 172.8             | | | 73.3  |
|               | >300     | 26 33.7%             | | | 852.5             | | | 552.2 |

NA, not applicable; SD, standard deviation.
The ALK patients. In two of the exposed female and 8.3% of exposed male ALK rearrangements in post-Chernobyl PTC samples 5

Table 2. Frequency of genetic alterations based on FISH and pyrosequencing

| Genetic alteration | Category | Unexposed (n = 19) | Exp (n = 77) | P value |
|--------------------|----------|-------------------|-------------|---------|
| ALK rearrangement frequency (FISH) | All Yes | 0 0.0 | 7 9.1 | |
| | No | 19 100.0 | 70 90.9 | 0.2 |
| | Females | Yes | 0 0.0 | 5 9.4 | |
| | No | 18 100.0 | 48 90.6 | 0.2 |
| | Males | Yes | 0 0.0 | 2 8.3 | |
| | No | 1 100.0 | 22 91.7 | 9.0 |
| ALK & RET rearrangement frequencies (FISH) | All Yes | 2 10.5 | 24 31.2 | |
| | No | 17 89.5 | 53 68.8 | 0.07 |
| | Females | Yes | 2 11.1 | 16 30.2 | |
| | No | 16 88.9 | 37 69.8 | 0.1 |
| | Males | Yes | 0 0.0 | 8 33.3 | |
| | No | 1 100.0 | 16 66.7 | 0.5 |

| Category | Unexposed (n = 19) | Exp (n = 76) | P value |
|----------|-------------------|-------------|---------|
| Point mutations (Kras, Nras, Braf) | All Yes | 6 31.6 | 21 27.6 | |
| | No | 13 68.4 | 55 72.4 | 0.7 |
| | Females | Yes | 5 27.8 | 15 28.9 | |
| | No | 13 72.2 | 37 71.1 | 0.9 |
| | Males | Yes | 1 100.0 | 6 25.0 | |
| | No | 0 0.0 | 18 75.0 | 0.1 |

| Category | Unexposed (n = 18) | Exp (n = 51) | P value |
|----------|-------------------|-------------|---------|
| RET rearrangement frequency (FISH) | All Yes | 2 11.1 | 17 33.3 | |
| | No | 16 88.9 | 34 66.7 | 0.07 |
| | Females | Yes | 2 11.1 | 11 33.3 | |
| | No | 16 88.9 | 22 66.7 | 0.08 |
| | Males | Yes | 0 0.0 | 6 33.3 | |
| | No | 1 100.0 | 12 66.7 | NA |

The frequency distributions remained (p = 0.07) after combining the ALK and RET rearrangements resulting in a total number of 26 translocations with a similar distribution (30.2–33.3%) among both genders (Table 2). In 95 examined biopsies, 27 point mutations [1 × Kras (Q61R), 1 × Kras (G13R), 1 × Nras (Q61R), 24 × Braf (V600E)] were detected in about one-third of exposed and unexposed females and males (p = 0.1–0.9; Table 2).

Association of biological endpoints with dose

About 50% of all Braf/Ras mutations were detected in the lowest dose category, and frequencies around 15–20% were observed at higher dose categories and in the unexposed group (Table 3). These differences were of borderline significance (p = 0.07–0.1; Table 3). ALK rearrangements showed a non-significant difference in frequency distribution. Almost 60% of the rearrangements were observed in the >100–500 mGy or the >60–300 mGy dose band (Table 3). RET rearrangements and combined rearrangements of the ALK and RET genes were statistically significant (p = 0.047) or of borderline significance (p = 0.06–0.1) as well as increased in frequency with increasing dose. The observed rearrangement frequencies increased about four-fold compared to the unexposed group (Table 3). These borderline significant associations remained in logistic regression analysis with adjustment for gender (but not for RAS mutations, data not shown) and ORs increased in a dose dependent manner, up to six-fold, and became significant (p = 0.04–0.05) after exposure >100 mGy when combining ALK and RET rearrangement frequencies (Table 4). Neither gender (adjusted or stratified) nor age at exposure or age at surgery contributed significantly to this association (data not shown). Also, these associations were consistent irrespective of the dose cut-point distributions used. Combined ALK and RET translocations showed a significant (p = 0.009) hazard ratio of 2.74 (95% CI: 1.29–5.83), to which gender did not contribute significantly (data not shown).

Immunohistochemistry

All FISH-positive ALK rearrangements were confirmed by IHC (strong homogenous ALK staining intensity of the tumour cells—score 2+ or 3+). Moreover, IHC staining of two FISH-negative cases highlighted aberrant expression of the ALK protein, eliciting a sensitivity of 100% and a specificity of 98% for the ALK antibody that was used in this study (clone 1A4). One of the two ALK-IHC+/alk-FISH− cases was derived seven ALK rearranged cases are illustrated in supplementary material, Table S1. The mean ages at incidence of ALK translocated patients (4.4 years) and of patients harbouring no ALK rearrangement (5.4 years) did not differ significantly (p = 0.91). The ALK rearrangement was found in about 9.4% of exposed female and 8.3% of exposed male patients. In two of the ALK+ cases, nuclear distribution of the ALK split and EML4 probe signals in FISH analysis supported a classification as an ALK-EML4 gene fusion (Figure 1). Among 69 examined biopsies, we detected 19 RET rearrangements in 33.3% of females and males of the exposed group, but only 11.1% of females of the unexposed group (p = 0.07–0.08; Table 2, right panel). These borderline significant differences in
from a patient who received a thyroid dose of 103 mGy and showed heterogeneous and weak staining intensity (score 1+). The second tissue sample, which originated from an unexposed patient, displayed strong but heterogeneous ALK staining of PTC cells (score 2+).

Discussion

In our study, we verified the presence of ALK rearrangements in a subset of post-Chernobyl patients who developed PTC after childhood exposure to radioiodine, but not among unexposed young Ukrainian PTC patients. In combination with the results for RET rearrangements, our findings confirm a significant association between calculated thyroid doses and the presence of gene rearrangements in PTCs, supporting results from previous studies [14,16,19,24,25]. On the contrary, point mutations of the BRAF and RAS genes that are frequently detected among sporadic adult PTCs were not associated with dose.

Previously, the prevalence of genetic alterations in PTC has been associated with age at diagnosis or...
surgery. In young PTC patients, mutually exclusive recombinations of genes like RET/PTC, NTRK1, PAX8/PPARγ, or AKAP9/BRAF represent the main oncogenic drivers, whereas BRAF or RAS point mutations were found in PTC at older age at onset [7]. However, the relevance of ALK rearrangements for increased risk of childhood PTC remains unclear. The largest assembly of PTC cases that had been studied so far showed an overall frequency of ALK rearrangements of 0.8%, but that study included mainly adults with a mean age of 46 years [8]. Interestingly, a recent study revealed five ALK gene alterations in 65 radiation-exposed PTC cases as shown by next-generation sequencing and RNA-seq (7.7%) with younger age at surgery, supporting our hypothesis of higher frequencies of ALK rearrangements in PTC among children with radiation aetiology [19].

Pyrosequencing analysis of 95 DNA samples extracted from PTC tissue revealed a total of 27 BRAF/RAS mutations (28.4%), of which BRAF-V600E mutations were most frequent (n = 24, 88.8%). PTC patients with BRAF/RAS mutations were older at the date of surgery (p = 0.01), in line with previous studies that showed a relationship between point mutations and age at diagnosis of PTC [8,14]. In our study, statistical analysis of a potential association between BRAF/RAS-positive tumours and dose failed to reach statistical significance. In summary, our findings strengthen the hypothesis that oncogenic point mutations are involved in the pathogenesis of sporadic PTC, which are frequently diagnosed at older ages, and lack a clear association with radiation-related PTC.

| Variable | Category (mGy) | All BRAF/RAS mutations | ALK rearrangements |
|----------|----------------|------------------------|--------------------|
|          | No (n = 68)    | Yes (n = 27)            | No (n = 89)        |
|          | n              | %                      | n                  |
|          | Chisq          | Chisq                  |                    |
| Dose categories |            |                        |                    |
| 0         | 13             | 19.1                   | 6                  |
| >0–100    | 16             | 23.5                   | 13                 |
| >100–500  | 24             | 35.3                   | 4                  |
| >500      | 15             | 22.1                   | 14                 |
|           |                |                        | 0.07               |
| 0         | 13             | 19.1                   | 6                  |
| >0–60     | 13             | 19.1                   | 11                 |
| >60–300   | 22             | 32.4                   | 4                  |
| >300      | 20             | 29.4                   | 6                  |
|           |                |                        | 0.10               |

| RET rearrangements | ALK and RET rearrangements |
|--------------------|-----------------------------|
| No (n = 50)        | Yes (n = 19)                |
| n                  | %                          | n | % | Chisq | n | % | Chisq |
| 0                  | 16                         | 32.0 | 2 | 8.5 | 17 | 24.3 | 2 | 7.7 |
| >0–100             | 16                         | 32.0 | 4 | 21.1 | 24 | 34.3 | 5 | 19.2 |
| >100–500           | 10                         | 20.0 | 7 | 36.8 | 18 | 25.7 | 11 | 42.3 |
| >500               | 8                          | 16.0 | 6 | 31.6 | 11 | 15.7 | 8 | 30.8 |
| 0                  | 16                         | 32.0 | 2 | 10.5 | 17 | 24.3 | 2 | 7.7 |
| >0–60              | 14                         | 28.0 | 4 | 21.1 | 20 | 28.6 | 4 | 15.4 |
| >60–300            | 9                          | 18.0 | 7 | 36.8 | 16 | 22.9 | 11 | 42.3 |
| >300               | 11                         | 22.0 | 6 | 31.6 | 17 | 24.3 | 9 | 34.6 |

| Chisq, Chi-square. |

| Table 3. Frequency distribution of mutations and gene rearrangements by dose categories |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Variable | Category (mGy) | All BRAF/RAS mutations | ALK rearrangements |                            |
|         |                | No (n = 68) | Yes (n = 27) | No (n = 89) | Yes (n = 7) | No (n = 70) | Yes (n = 26) |                            |
|         |                | n | % | n | % | n | % | n | % | Chisq | n | % | n | % | Chisq |
| Dose categories |            |              |              |              |              |              |              |              |              |                    |              |              |              |              |                    |
| 0         | 13             | 19.1 | 6 | 22.2 | 19 | 21.4 | 0 | 0.0 |              |              |                    |              |              |              |              |                    |
| >0–100    | 16             | 23.5 | 13 | 48.2 | 28 | 31.5 | 1 | 14.3 |              |              |                    |              |              |              |              |                    |
| >100–500  | 24             | 35.3 | 4 | 14.8 | 25 | 28.1 | 4 | 57.1 |              |              |                    |              |              |              |              |                    |
| >500      | 15             | 22.1 | 14 | 48.2 | 17 | 19.1 | 2 | 28.6 | 0.07 | 17 | 19.1 | 2 | 28.6 | 0.07 |                    |
| 0         | 13             | 19.1 | 6 | 22.2 | 19 | 21.4 | 0 | 0.0 |              |              |                    |              |              |              |              |                    |
| >0–60     | 13             | 19.1 | 11 | 40.7 | 24 | 27.0 | 0 | 0.0 |              |              |                    |              |              |              |              |                    |
| >60–300   | 22             | 32.4 | 4 | 14.8 | 23 | 25.8 | 4 | 57.1 |              |              |                    |              |              |              |              |                    |
| >300      | 20             | 29.4 | 6 | 22.2 | 23 | 25.8 | 3 | 42.9 | 0.10 | 23 | 25.8 | 3 | 42.9 | 0.10 |                    |

| Table 4. Association of rearrangements (ALK and RET) with radiation dose among PTC cases after the Chernobyl accident |
|---------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Model                          | Dose categories | OR   | 95% CI | P value |                            |
| ALK and RET rearrangements    | 0               | 1.00 | 0.00  | 1.00  |                            |
| >0–100                        | 1.8             | 0.3  | 10.2  | 0.53  |                            |
| >100–500                      | 5.2             | 1.0  | 26.9  | 0.050 |                            |
| >500                           | 6.2             | 1.1  | 34.7  | 0.039 |                            |
| ALK and RET rearrangements    | 0.06            |       |       |       |                            |
| >0–60                         | 1.7             | 0.3  | 10.4  | 0.57  |                            |
| >60–300                       | 5.8             | 1.1  | 30.5  | 0.036 |                            |
| >300                          | 4.5             | 0.8  | 24.0  | 0.078 |                            |
| CI, confidence interval; OR, odds ratio.                        |
RET-FISH revealed 19 RET rearrangements out of 69 analysable cases (27.5%). Non-analysable/missing tissue samples were mainly from exposed individuals, so this may be an under-estimate of the effect of ionizing radiation. Nevertheless, our findings align with the expectation that RET gene fusions are frequently detected in sporadic and to an even higher extent in radiation-related childhood PTC [7,14,19].

Three-colour FISH analysis of PTC tissue samples revealed ALK rearrangements in exposed cases only. Indeed, the 7 identified ALK-rearranged cases (out of 77 exposed patients, 9.1%) were observed among those with individual thyroid doses from 62 mGy up to 1085 mGy, for which a borderline significant frequency distribution was seen (p = 0.10). Of note, in two of the FISH-positive cases, the ALK-EML4 split/fusion signals were so distinct that we classified these cases as harbouring an ALK-EML4 inversion. This type of rearrangement has been previously detected in aggressive BRAF-wild type PTC and, interestingly, the patient in that report had undergone radioiodine therapy and external beam radiation [26]. In the other cases, a distinction between ALK-EML4 or the previously reported ALK-STRN rearrangement could not be verified based on FISH analysis alone [15]. This limitation is due to the relatively close proximity of the EML4 and STRN genes on the short arm of chromosome 2. To assess the role of ALK IHC in the detection of ALK gene rearrangement, we also performed IHC using a commercial ALK-antibody (clone 1A4) which is in diagnostic use for routine ALK testing in lung cancer [23]. IHC staining of the ALK receptor tyrosine kinase confirmed the findings from ALK-FISH and provided no false-negative results. However, two FISH-negative cases had to be considered positive for ALK staining, represented by one borderline case from an exposed patient and a second clearly ALK-positive tissue sample from a patient lacking radiation aetiology. This may not be unexpected since a recent study demonstrated discordant FISH results for NSCLC cases with positive ALK IHC staining [27]. This may be due to the fact that aberrant ALK protein expression as shown by ALK IHC may not solely be ascribed to genetic alterations such as gene fusion or translocation/inversion events, but also to various pathological modifications along mutational, epigenetic, splicing, or transcriptional processes. Interestingly, beneficial ALK inhibitor treatment was reported in a FISH negative and IHC positive NSCLC patient, demonstrating the clinical relevance of ALK IHC screening [28].

Regarding the confirmed association between ALK rearrangements and previous exposure to ionizing radiation, our findings suggest ALK IHC as a suitable and cost-effective screening method for potential drug targeting of ALK rearrangements, particularly among PTC patients with former therapeutic or accidental radiation exposure. Even for sporadic PTC where ALK rearrangements have been shown to display low-frequency occurrence, ALK IHC testing of thyroid cancer cases which fail to respond to conventional therapy like surgery or I-131 treatment might be advisable.

Our findings support the hypothesis that radiation-related gene fusions trigger the pathogenesis of childhood PTC. In our study of PTC tissue samples after the Chernobyl accident, including a non-exposed group, we confirmed an association between rearrangements of the ALK and RET genes, but not for BRAF or RAS point mutations with thyroid gland exposure to I-131. Taking into account that ALK-targeted therapy is clinically available, ALK IHC may offer a cost-effective screening method especially among PTC patients with known radiation exposure and resistance to conventional therapy.

Acknowledgements

The authors gratefully acknowledge the confirmation of diagnosis provided by the International Pathology Panel of the Chernobyl Tissue Bank: A. Abrosimov, T. Bogdana\-nova, N. Dvinskikh, G. Fadda, J. Hunt, M. Ito, V. LiVolsi, J. Rosai, E. Williams. The authors thank Dr. Zurnadzhy and Dr. Voskoboinyk for providing the FFPE samples, Dres Puchkarov for preparing the DNA samples, the staff of the Imperial College for excellent organisation and Claudia Schlosser as well as Wiebke Hemmer of the Institute of Pathology and Molecular Pathology, Bundeswehrkrankenhaus Ulm for expert technical assistance. The CTB is supported by grants from the National Cancer Institute (NCI; 5U24CA082102) and the SMHF of Japan. The project was supported by the Qiagen team for Personalized Healthcare (PHC).

Author contributions statement

AA and KS carried out data acquisition and data analysis, helped to conceive the study and helped to draft the manuscript. AR and MP helped to conceive the study and helped to draft the manuscript. MS manufactured the TMA. TB provided pathological diagnosis and marked the pathology sections to make
the TMA. LK calculated thyroid dose estimates. MA helped to draft the manuscript and carried out statistical analysis. SE helped to conceive the study, helped to carry out statistical analysis and drafted the manuscript. The final manuscript was read and approved by all authors.

References

1. Schneider AB, Sarne DH. Long-term risks for thyroid cancer and other neoplasms after exposure to radiation. Nat Clin Pract Endocrinol Metab 2005; 1: 82–91.

2. Cardis E, Kesminiene A, Ivanov V, et al. Risk of thyroid cancer after exposure to 131I in childhood. J Natl Cancer Inst 2005; 97: 724–732.

3. Brenner AV, Tronko MD, Hatch M, et al. 1-131 dose response for incident thyroid cancers in Ukraine related to the Chernobyl accident. Environ Health Perspect 2011; 119: 933–939.

4. Williams D. Radiation carcinogenesis: lessons from Chernobyl. Oncogene 2008; 27: S9–S18.

5. Pacini F, Vorontsova T, Demidchik EP, et al. Post-Chernobyl thyroid carcinoma in Belarus children and adolescents: comparison with naturally occurring thyroid carcinoma in Italy and France. J Clin Endocrinol Metab 1997; 82: 3563–3569.

6. Suzuki K, Saenko V, et al. Radiation signatures in childhood thyroid cancers after the Chernobyl accident: possible roles of radiation in carcinogenesis. Cancer Sci 2015; 106: 127–133.

7. Yamashita S, Saenko V. Mechanisms of disease: molecular genetics of childhood thyroid cancers. Nat Clin Pract Endocrinol Metab 2007; 3: 422–429.

8. Agrawal N, Akbani R, Aksoy BA, et al. Integrated genomic characterization of papillary thyroid carcinoma. Cell 2014; 159: 676–690.

9. Fenton CL, Lukeys Y, Nicholson D, et al. The ret/PTC mutations are common in sporadic papillary thyroid carcinoma of children and young adults. J Clin Endocrinol Metab 2000; 85: 1170–1175.

10. Bongarzone I, Fugazza L, Vigneri P, et al. Age-related activation of the tyrosine kinase receptor protooncogenes RET and NTRK1 in papillary thyroid carcinoma. J Clin Endocrinol Metab 1996; 81: 2006–2009.

11. Sassolas G, Hafdi-Nejjari Z, Ferraro A, et al. Oncogenic alterations in papillary thyroid cancers of young patients. Thyroid 2012; 22: 17–26.

12. Penko K, Livezey J, Fenton C, et al. BRAF mutations are uncommon in papillary thyroid cancer of young patients. Thyroid 2005; 15: 320–325.

13. Rosenbaum E, Hosler G, Zahurak M, et al. Mutational activation of BRAF is not a major event in sporadic papillary thyroid carcinoma. Mod Pathol 2005; 18: 898–902.

14. Leeman-Neill RJ, Brenner AV, Little MP, et al. RET/PTC and PAX8/PPARγ chromosomal rearrangements in post-Chernobyl thyroid cancer and their association with iodine-131 radiation dose and other characteristics. Cancer 2013; 119: 1792–1799.

15. Kelly LM, Barila G, Liu P, et al. Identification of the transforming STRN-ALK fusion as a potential therapeutic target in the aggressive forms of thyroid cancer. Proc Natl Acad Sci U S A 2014; 111: 4233–4238.

16. Hamatani K, Mukai M, Takahashi K, et al. Rearranged anaplastic lymphoma kinase (ALK) gene in adult-onset papillary thyroid cancer amongst atomic bomb survivors. Thyroid 2012; 22: 1153–1159.

17. Werner MT, Zhao C, Zhang Q, et al. Nucleophosmin-anaplastic lymphoma kinase: the ultimate oncogene and therapeutic target. Blood 2017; 129: 823–831.

18. Pérot G, Soubeynan I, Ribeiro A, et al. Identification of a recurrent STRN/ALK fusion in thyroid carcinomas. PloS One 2014; 9: e87170.

19. Efano AA, Brenner AV, Bogdanova TI, et al. Investigation of the relationship between radiation dose and gene mutations and fusions in post-Chernobyl thyroid cancer. J Natl Cancer Inst 2011; 103: 371–378.

20. Kruczyński A, Delso G, Laurent C, et al. Anaplastic lymphoma kinase as a therapeutic target. Expert Opin Ther Targets 2012; 16: 1127–1138.

21. Likhtarov I, Thomas G, Kovygan L, et al. Reconstruction of individual thyroid doses to the Ukrainian subjects enrolled in the Chernobyl Tissue Bank. Radiat Prot Dosimetry 2013; 156: 407–423.

22. Likhtarov I, Bouville A, Kovygan L, et al. Questionnaire- and measurement-based individual thyroid doses in Ukraine resulting from the Chernobyl nuclear reactor accident. Radiat Res 2006; 166: 271–286.

23. Gruber K, Kohlhäusl M, Friedel G, et al. A novel, highly sensitive ALK antibody 1A4 facilitates effective screening for ALK rearrangements in lung adenocarcinomas by standard immunohistochemistry. J Thorac Oncol 2015; 10: 713–716.

24. Hamatani K, Eguchi H, Ito R, et al. RET/PTC rearrangements preferentially occurred in papillary thyroid cancer among atomic bomb survivors exposed to high radiation dose. Cancer Res 2008; 68: 7176–7182.

25. Leeman-Neill RJ, Kelly LM, Liu P, et al. ETV6-NTRK3 is a common chromosomal rearrangement in radiation-associated thyroid cancer. Cancer 2014; 120: 799–807.

26. Demeure MJ, Aziz M, Rosenberg R, et al. Whole-genome sequencing of an aggressive BRAF wild-type papillary thyroid cancer identified EML4-ALK translocation as a therapeutic target. World J Surg 2014; 38: 1296–1305.

27. Paik JH, Choe G, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer: correlation with fluorescence in situ hybridization. J Thorac Oncol 2011; 6: 466–472.

28. Sun J-M, Choi Y-L, Won J-K, et al. A dramatic response to crizotinib in a non-small-cell lung cancer patient with IHC-positive and FISH-negative ALK. J Thorac Oncol 2012; 7: e36–e38.

29. Bastos AU, de Jesus AC, Cerutti JM. ETV6-NTRK3 and STRN-ALK kinase fusions are recurrent events in papillary thyroid cancer of adult population. Eur J Endocrinol 2018; 178: 85–93.

SUPPLEMENTARY MATERIAL ONLINE

Table S1. Characteristics of patients harbouring ALK rearrangements