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Genetic characterization of medullary thyroid cancer in childhood survivors of the Chernobyl accident ☆,☆☆

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ABSTRACT

Background: Radiation-associated fusion oncogenes play a direct role in papillary thyroid cancer development and pathogenic fusions have recently been reported in medullary thyroid cancer. To date, no studies have evaluated oncogenic events in medullary thyroid cancer in a radiation-exposed population.
Methods: Somatic and germline alterations, including *RET* fusions, were evaluated in paired medullary thyroid cancer tumor and normal samples from the Chernobyl Tissue Bank, a heavily screened population affected by the Chernobyl disaster.
Results: Tissue was available for 49 individuals. The median age of diagnosis was 26 years (range 9 to 43 years); 16 were radiation-exposed at a median age of 6 (range 2 days to 17 years). A total of 21 patients harbored germline *RET* mutations (codons 634[13], 918[5], 790[1], 609[1], and 620[1]); 4 had family history. Sporadic medullary thyroid cancer was identified in 27 patients (*RET*[18], *KRAS*[1], *RET*+*KRAS*[1], *TP53*[1], wild type [6]), with 1 *RET* fusion (1/49;2%). The age at operation for patients with hereditary medullary thyroid cancer was not different than sporadic medullary thyroid cancer (23.5 vs 28 years, $P = .063$). In sporadic medullary thyroid cancer, radiation was not associated with a difference in age at operation, tumor size, or tumor stage ($P > .05$).
Conclusion: In a heavily screened cohort, genetic analysis revealed germline *RET* mutations in previously unrecognized probands and a remarkable number of sporadic medullary thyroid cancer cases with a young age at presentation.

Introduction

Medullary thyroid cancer (MTC) is a rare malignancy (1–2% of all thyroid cancers) that arises from calcitonin-producing parafollicular C cells.[1] Of the 25% of patients in whom MTC is hereditary, >95% demonstrate germline activating point mutations of *RET* (rearranged during transfection). For the remaining 75% of patients with sporadic MTC, activating mutations of *RET* have been identified in only 40%–50%, with activating *RAS* mutations accounting for another 10–15%, leaving a substantial number of patients without an identified driver mutation.[1] In the era of genomics this gap in

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knowledge represents significant potential for discovery with therapeutic impact.

In contrast to MTC, in patients with papillary thyroid cancer (PTC), studies of exposure to either therapeutic or environmental radiation (ie, from a natural disaster such as the 1986 Chernobyl nuclear reactor accident) have established strong evidence for the dose-dependent role of ionizing radiation in cancer development, often mediated through fusion oncogenes.[2] Patients with PTC and radiation exposure are more likely to harbor *RET* fusion oncogenes as compared to their unexposed counterparts. In 1 study of young Ukrainian patients with PTC who were exposed as children to nuclear fallout from the 1986 Chernobyl accident, known *RET* fusions accounted for driver mutations in 18 of the 26 patients, and 3 additional novel fusion oncogenes were identified; fusions were not as prevalent in a similar cohort of unexposed patients.[3] Although fusion oncogenes have been well characterized in the PTC population, only recently have they been described in MTC.[4,5] MTC occurring in patients exposed to radiation has not been well characterized. In particular, the role of radiation and its relationship to fusion oncogenes in patients with MTC is unknown.

In the current study we describe a cohort of patients with MTC from the affected regions of the Chernobyl disaster and genetically characterize their tumors. We hypothesized that radiation exposure could be associated with the development of fusion oncogenes in MTC.

Methods

The Chernobyl Tissue Bank[6] (CTB) is an international research collaboration that collects and stores normal and tumor tissue from patients with thyroid abnormalities from the contaminated oblasts of the Russian Federation and Ukraine who were less than 19 years of age at the time of the Chernobyl nuclear disaster (April 26, 1986) and operated on or after October 1, 1998 (the date of the establishment of the collaboration). Tissue from patients born after April 26, 1986 and/or not directly exposed to the nuclear fallout but who lived in similar geographic areas is also included in the bank for comparison. Exposure was determined directly by the CTB. After Institutional Review Board approval, the CTB was queried for all patients with MTC. All clinical information was received in deidentified format, in concordance with the Health Insurance Portability and Accountability Act of 1996. Clinical variables including age at time of operation, initial staging, and exposure to radioactive fallout were collected directly from the CTB.

Next-generation sequencing (NGS) using the Ion Torrent Ampliseq50 panel was performed on DNA extracted from formalin-fixed, paraffin-embedded tissue sections. Library preparation, sequence alignment, variant calling, and variant review were performed as described previously[7] per established protocols; samples that failed NGS underwent targeted Sanger sequencing of *RET* exons 10, 11, 13–16 and *H/KRAS* codons 12, 13, and 61. To differentiate between hereditary and sporadic cancers, targeted Sanger sequencing of all identified *RET* tumor DNA mutations was also performed on normal thyroid tissue from the same patient. In cases in which normal thyroid tissue was inadequate, banked blood samples were used. Break-apart fluorescence in situ hybridization (FISH) was performed to evaluate for *RET* rearrangements using the commercially available dual color probe set, Clear-View™ FISH RET Break Apart Probe (CymoGen Dx). Slides were deparaffinized in citrisolv, washed in ethanol, and pretreated with SPoT-Light™ Tissue Pretreatment Kit (Invitrogen) at 98°C for 33 minutes. Tissues were subsequently digested using the SPoT-Light™ tissue pretreatment enzyme for 25 minutes at room temperature, washed in a solution of 2x saline sodium citrate with 0.025% Tween-20 (Sigma-Aldrich), and dehydrated with graded ethanol before hybridization with denatured *RET* break apart probe. Hybridization was performed over 2 nights at 37°C after DNA denaturation at 85°C for 10 minutes. Subsequently, slides were washed at 65°C for 20 minutes, dehydrated with graded ethanol, and then counter-stained with 4',6-diamidino-2-phenylindole (DAPI).

Hematoxylin and eosin stained slides were used to identify tumor tissue, and FISH analysis was performed on these sections with a minimum of 200 nuclei analyzed by 2 independent reviewers. The cutoff for break-apart positivity was defined as ≥8.17% of nuclei with break-apart signal (2 standard deviations above the mean assessed on normal thyroid tissue). Specimens meeting the initial cutoff were then reviewed by 2 independent cytogeneticists for confirmation. Dual-indexed NGS libraries with molecular barcoding were prepared from 100ng total formalin-fixed, paraffin-embedded (FFPE) tumor RNA using a custom-designed anchored multiplex polymerase chain reaction (PCR)-based assay (ArcherDX, Inc.) targeting 485 exons in 81 genes recurrently involved in solid tumor fusions. RNA libraries were multiplexed and sequenced on an Illumina MiSeq (2 × 150bp), and data analysis for deduplication and fusion detection were performed on a virtual-machine hosted RNA pipeline (Archer Analysis).

Statistical Package for the Social Sciences 20.0 (IBM, Armonk, NY) was used for analysis. Comparisons between continuous variables were made with Student's *t*-test. Comparisons between categorical variables were made with Pearson chi-square, Fisher's exact test, or analysis of variance as appropriate for the data.

Table
Clinical and pathologic characteristics of patients undergoing surgery for medullary thyroid cancer from the Chernobyl Tissue Bank ($n = 49$)*

	n (%) or median (range)
Female sex	35 (71.4)
Radiation exposure from nuclear fallout	16 (32.7)
Age at time of Chernobyl†	6.4 (2 days–17.2 years)
Age at time of initial operation (years)	26.2 (9.0–42.6)
Tumor size (mm)	21.5 (0.6–65.0)
Stage‡	
I	9 (18.4)
II	10 (20.4)
III	22 (44.9)
IVa	3 (6.1)
IVb	0
IVc	1 (2.0)

* with adequate tumor tissue available
**Calculated only for those exposed to Chernobyl ($n = 16$)
‡ AJCC 7th edition, at the time of operation; stage unknown $n = 4$, 8.2%

Results

The CTB currently includes clinical data and/or biologic samples from 4,500 patients who have undergone thyroid surgery at 1 of 2 referral hospitals in the Ukraine and Russian Federation since October 1, 1998.[6] Of these, 3,232 operations were performed for thyroid cancer, and 82 (2.5%) had MTC. A total of 50 patients had banked tissue available for analysis; pathologic review noted 1 patient without tumor identified in the provided material and the CTB confirmed it had no additional tissue—this patient was excluded from all analyses, leaving a study population of 49 patients with available tumor tissue. The clinical and pathologic characteristics of these patients are summarized in the Table. Figure 1 demonstrates the geographical distribution of the study population in comparison with the estimated pattern of radiographic fallout.[8]

Genetic characterization

In total, 5 patients had indeterminate results of genetic testing (Fig 2). Of these, 1 had an M918T *RET* mutation in the tumor and

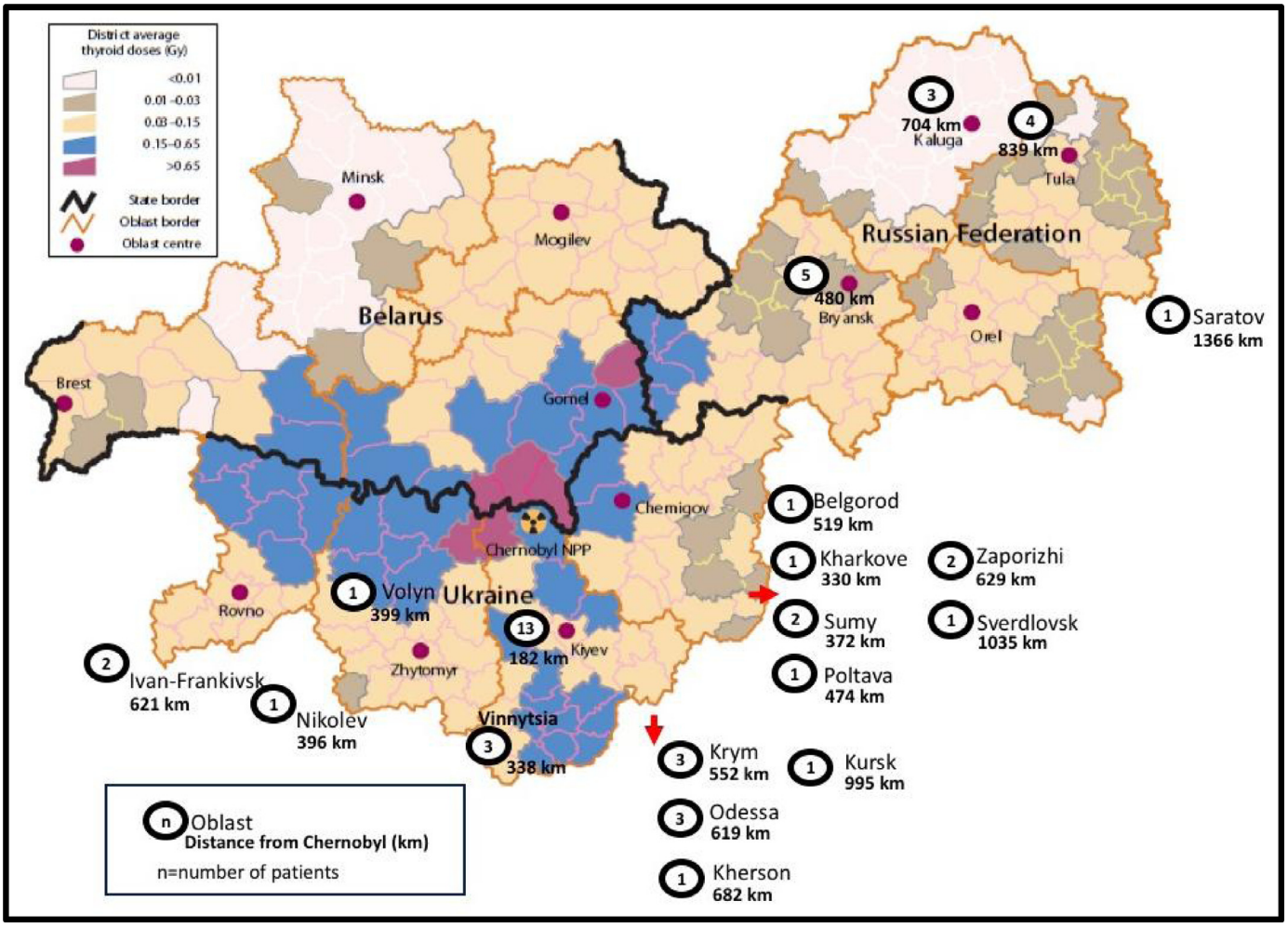

Fig. 1. Distribution of study subjects in relation to the estimated fallout from Chernobyl.
Source: Adapted with permission from the United Nations Scientific Committee on the Effects of Atomic Radiation; UNSCEAR 2008 Report to the General Assembly, with scientific annexes.

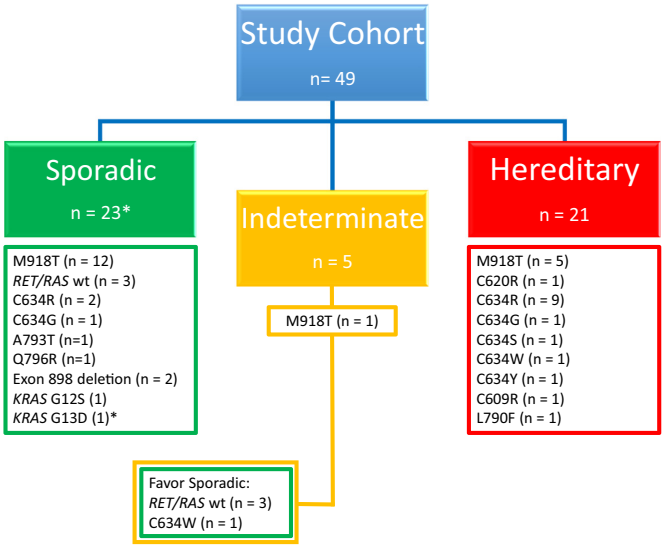

Fig. 2. Schematic of somatic and germline mutations.
**KRAS* mutation identified in a patient with a concurrent M918T mutation, total $n = 23$. *wt*, wild type

inadequate normal tissue and did not have banked blood samples; this patient was excluded from all analyses examining sporadic versus hereditary MTC. There were 3 patients who failed NGS and had incomplete Sanger sequencing—these patients were wild type in each *RET* exon except for the failed exons (exon 14 and 15, exon 11 and 15, and exon 10 and 11) and were assumed to represent sporadic disease. One patient had a *RET* C634W mutation in the tumor DNA; however, normal tissue failed testing. As sequencing signal was consistent with a sporadic mutation, this patient was counted as sporadic in all analyses.

Hereditary medullary thyroid cancer

Germline mutational analysis identified 21 patients with hereditary MTC (42.9%); 5 (23.8%) with MEN2B as defined by the p.Met918Thr alteration in exon 16, and 16 with MEN2A (p.Cys634Arg $n = 9$, p.Cys634Gly $n = 1$, p.Cys634Ser $n = 1$, p.Cys634Trp $n = 1$, p.Cys624Tyr $n = 1$, p.Cys620Arg $n = 1$, p.Cys609Arg $n = 1$, and p.Leu790Phe $n = 1$). The distribution of MEN2A mutations is shown in Figure 3 in comparison with historical studies. Of the patients with hereditary MTC, the median age at operation was 23.2 years (range 9.0–39.2 years). Seventeen of the patients represented the family proband; 4 had a documented family history of MTC. In the 21 patients with hereditary disease, the median tumor size was 25.3 mm (range 5.0 to 60.0 mm). Stage at operation included 4 with stage I disease, 5 with stage II,

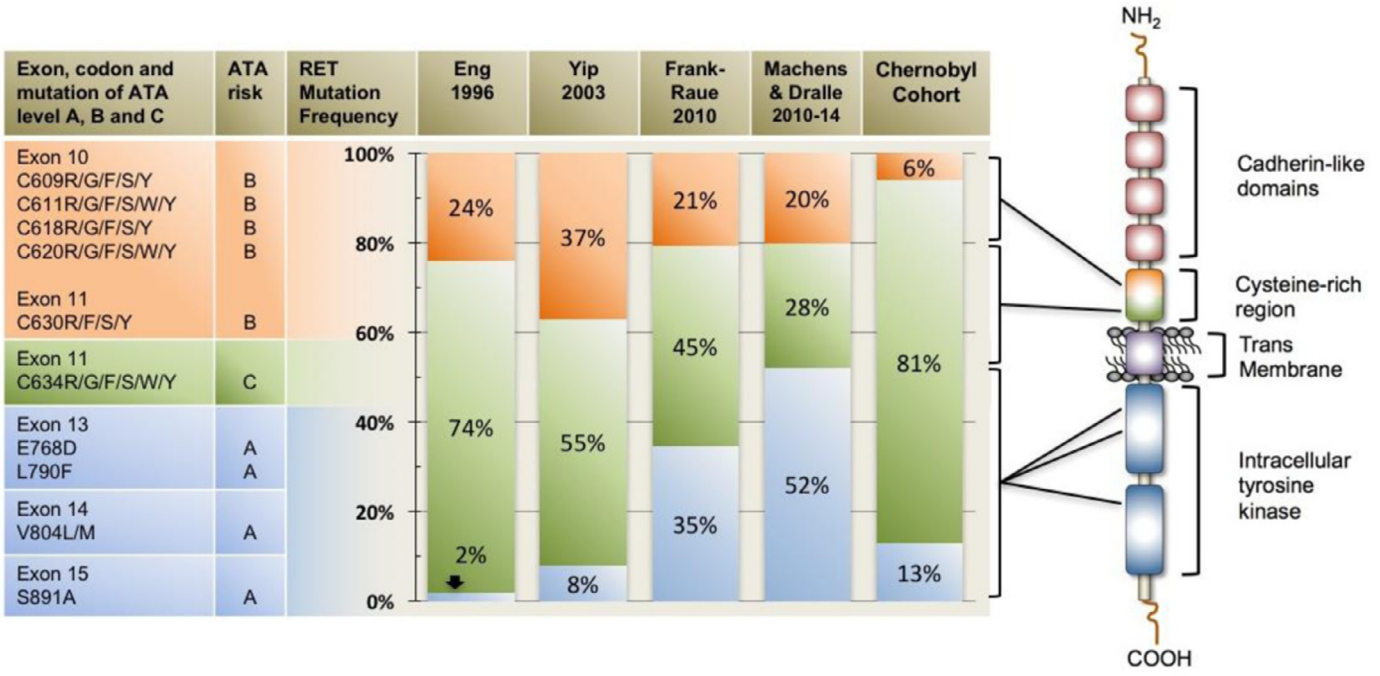

Fig. 3. Distribution of driver mutations in MEN2A patients from the Chernobyl region.
Source: Adapted with permission from Grubbs and Gagel, JCEM 2005.

10 with stage III, 1 with stage IVa, and 1 patient with stage IVc owing to liver metastases. The 5 patients with MEN2B presented with stage II ($n = 2$) and III ($n = 3$) disease at an average age of 17.8 years, as compared to 25.3 years for the others with hereditary disease ($P = .080$).

Sporadic medullary thyroid cancer

For the 27 patients with sporadic MTC, the median age at operation was 28.9 years (range 13.9–42.6 years). A family history of MTC was reported by 2 patients, although specific details are unknown; 1 had a p.Cys634Gly alteration present in tumor DNA but not in normal thyroid DNA, and the other was *RET*/*RAS* wild type. The median tumor size was 19.0 mm (range 0.6–65.0 mm). Mutation status is summarized in Figure 2 and includes 20 patients with *RET* mutations, 2 with *RAS* mutations (1 of whom had a concurrent *RET* mutation), and 6 who were wild type for *RET* and *RAS*. Stage at operation included 5 patients with stage I disease, 5 with stage II, 11 with stage III, and 2 with stage IVa disease; 4 had insufficient information for staging.

Comparison of patients with sporadic versus hereditary medullary thyroid cancer

The age at operation was not statistically different between patients with sporadic versus hereditary MTC (28.0 years vs 23.5 years, $P = .063$). Tumor size was similar between patients with sporadic and hereditary MTC (25.5 cm vs 28.1 cm, $P = .634$). Tumor stage at operation was also not different between patients with sporadic versus hereditary MTC ($P = .670$).

Radiation exposure

Of the 16 patients who were exposed to radioactive fallout from Chernobyl, the median age at exposure was 6.4 years (range 2 days–17.2 years), and the median age at operation was 21.2 years (range 15.0–26.6 years). There were no significant differences in age at operation, tumor size, or stage at operation for patients who were exposed versus unexposed (mean age 28.7 years vs 24.4 years, $P = .093$; mean tumor size 23.7 vs 29.0 mm, $P = .380$; $P = .383$ for stage). There were no observed differences in mutational profiles between radiation-exposed and unexposed patients ($P = .13$). Eleven patients (40.7%) with sporadic MTC were exposed to radioactive fallout from Chernobyl. When limited to patients with sporadic MTC only, there were no significant differences between age at operation, tumor size, or stage at operation for patients who were exposed versus unexposed (26.7 years vs 29.0 years, $P = .465$; 25.4 mm vs 25.5 mm, $P = .990$; $P = .970$ for stage).

RET *chromosomal rearrangements*

FISH for break-apart *RET* rearrangements was performed successfully on 44 tumor samples (4 had insufficient tumor to perform analyses and 1 failed to hybridize). Consensus review confirmed *RET* rearrangement in 1 patient (Fig 4). Sequencing demonstrated that this patient had a p.Met918Thr alteration; a banked blood sample confirmed that this was not a germline mutation. Targeted RNA NGS failed to identify a *RET* fusion partner; however, the analysis was inconclusive owing to insufficient RNA sequencing reads. The patient was 18 years old at the time of operation, was exposed to fallout from Chernobyl at the age of 2, had no known family history of MTC, and presented with a 0.6 cm T1N0M0 tumor.

Discussion

The current study characterizes the driver mutation profiles of Chernobyl survivors with MTC. Although thyroid cancer survivors are well studied from this region, the majority of the focus to date has been on those with a diagnosis of papillary thyroid cancer.[2,3,9–15] To our knowledge the current study represents the first directed examination of MTC in an irradiated population.

After the Chernobyl accident, the incidence of thyroid cancer in the affected regions rose dramatically. It was first recognized internationally as a public health concern in 1992.[9,16] Several national

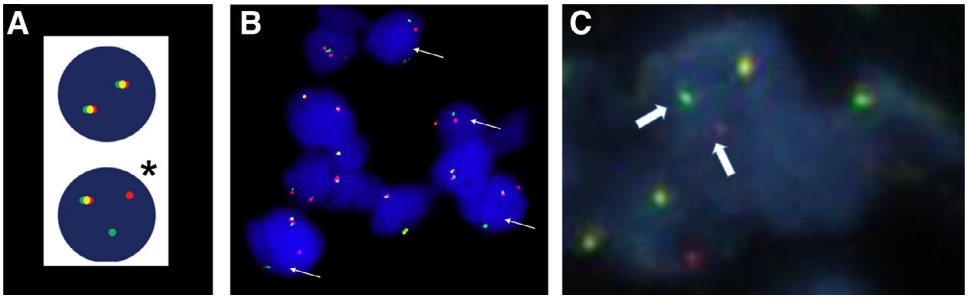

Fig. 4. Positive *RET* break-apart FISH, with the diagram in (A) demonstrating separation of the probes (*), (B) as performed on recently banked tissue from our institution, and as compared to (C) tissue banked after the Chernobyl accident.
Source: Adapted with permission from Grubbs et al, JCEM 2015.

registries to identify affected patients and 2 large population-based screening programs were developed concomitantly.[10,13,15] The impact of the screening programs on the identified incidence of MTC is unclear. Of available estimates regarding post-Chernobyl thyroid cancers, MTC comprises between 2.4% and 6% of all thyroid cancers occurring in the region,[12,14,17] which is similar to our study population. The age at operation, and presumed diagnosis, in the current study is remarkably younger in patients with sporadic MTC (median 28.9 years) than what is typically observed in Western populations (presentation in the fifth decade).[18] This observed young age can be attributed, at least in part, to the intensive screening programs adopted in this region, in particular targeting patients who were exposed as children to Chernobyl radiation. By definition, the CTB collects tissue only from patients who were 0–19 years of age at the time of Chernobyl, which additionally limits the cohort to patients less than 51 years of age currently.

The current study includes a large number of patients with germline *RET* mutations and presumed multiple endocrine neoplasia 2 (MEN2). It is notable that of the 21 patients with MEN2, only 4 had a documented family history. This finding may be accounted for, in part, by the unexpectedly high proportion of patients with MEN2B in our study (23% of all hereditary cases), which most often arises *de novo*. A lack of thorough family history may have contributed to these results (clinical data across a 30-year period within a large, often politically unstable region was fragmented). Another potential explanation for the unusually high number of probands may be related to the screening efforts in this region post-Chernobyl, although the median tumor size and moderately advanced stage at operation for patients with both hereditary and sporadic MTC suggests that disease was often identified clinically. One of the challenges of utilizing the CTB data is that the patients included were not all identified through the screening programs but instead were a mix of screened and clinically apparent cases, with no denotation of how each was diagnosed. Interestingly, of the patients with MEN2A, the distribution of identified mutations is not what one would expect with modern screening techniques, with the majority of patients demonstrating mutations in codon 634, or American Thyroid Association level C/high risk (Fig 3), the so-called noisy phenotype. This is in contrast to what one would expect in a heavily screened population[19,20] and more consistent with historical data that reflects a time when most patients were identified clinically.[21,22] With limited and deidentified clinical records we are unable to determine whether any of the patients harboring the same germline mutation were kindred.

For patients with sporadic MTC, p.Met918Thr in exon 16 is the most frequently reported mutation and is the most frequently observed in in the current study.[23] It is of interest, however, that *RET* or *KRAS* mutations were identified in 88% of patients with sporadic MTC. This is substantially higher than expected, with the COSMIC database and other studies in the literature identifying driver mutations in only 40%–60% of sporadic MTC.[1,23] One plausible explanation may be the use of NGS in the current study that allows more comprehensive profiling. Others have also shown improved genetic characterization with NGS, with driver mutations demonstrated in 85%–90% of sporadic MTC samples.[24,25]

MTC has historically been thought to develop independent of the effects of radiation, in part because of its low mitotic rate of parafollicular C cells and hence decreased susceptibility to genetic breakage during replication.[26] Yet the close anatomic relationship of these cells to the radiosensitive follicular cells of the thyroid known to preferentially take up I^{131} prompts the question of secondary effects of radiation. Minimal research exists examining the effects of radiation, and specifically I^{131}, on MTC. In animal models I^{131} induced maternal hypothyroidism both before pregnancy and during early gestation led to hyperplasia and hypertrophy of calcitonin expressing cells in the thyroids of the offspring.[27] Whether this is due to the maternal hypothyroidism or direct effects of the I^{131} is unclear. In a small study of patients exposed to therapeutic radiation during infancy, peritumoral C-cell hyperplasia, a neoplastic precursor to MTC, was identified at a higher rate in patients with thyroid tumors exposed to radiation (55%) as opposed to those without radiation exposure (7%) or in control normal thyroid tissue (10%).[11] We hypothesized that *RET* fusions may be more likely to be discovered in MTC tumors exposed to radiation than in those not exposed but found that *RET* fusions occurred infrequently in this MTC population and were not associated with radiation exposure. In addition, radiation-exposed patients did not undergo operation at a demonstrably earlier age or more advanced stage than their unexposed counterparts. These findings add to the theory that MTCs develop independently of the effects of radiation.

We identified 1 patient with a *RET* fusion oncogene. Well characterized in PTC, fusion oncogenes have only recently been identified in patients with MTC.[5] We recently described a patient with aggressive metastatic MTC with a *MYH13-RET* fusion who presented with advanced disease at a young age (46).[4] The CTB tissue procurement practices during the collection period are unknown but robust tissue procurement, processing, and archiving procedures are required to ensure that RNA extracted from FFPE tissue is suitable for RNA-based diagnostics. Although we attempted to identify the *RET* fusion partner in the *RET*-rearranged case using Archer NGS, the low total and unique number of RNA reads that were generated suggested insufficient usable RNA was extracted from the FFPE sections.

This study has several limitations. Clinical follow-up across a politically fragmented region over a time course of more than 30 years is inherently difficult. The patients in this study comprise both those who presented symptomatically as well as many who were identified during rigorous screening programs, and the CTB is not designed to identify the mechanism of presentation. Within the hereditary group we are unable to discern which patients un-

derwent prophylactic versus therapeutic operations. As a tissue repository, the CTB is also not designed to maintain longitudinal data, and records of clinical outcomes for patients are limited, which prevents examination of clinicopathologic parameters of disease aggressiveness. Radiation exposure was a variable provided by the CTB; we are unable to examine in utero exposure because dates of birth were not provided. Tissue artifact of FFPE slides also complicated FISH interpretation. Finally, there were 5 patients with incomplete genetic analysis, 1 of whom was indeterminate and excluded from all comparative analyses, and 4 of whom were suspected to have sporadic disease. These 4 patients were included in comparison analyses between sporadic and hereditary populations and could have introduced bias.

This study identifies a unique cohort of patients with MTC, including a subset of patients with sporadic MTC presenting at an unusually young age, which may be related to post-Chernobyl screening efforts in the region, and a surprising number of patients with MEN2B. The use of next generation sequencing increased the proportion of identified driver mutations, and FISH for *RET* rearrangements demonstrated 1 suspected *RET* fusion. Although the present study is small, no differences were observed in age or stage at operation, or tumor size between those exposed to radiation versus unexposed, suggesting that MTC development is independent of radiation exposure. The current study is the first of its kind to genetically characterize a cohort of patients with MTC from the Ukraine and Russian Federation impacted by the 1986 Chernobyl disaster.

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Conflicts of interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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