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TRIOLOGICAL SOCIETY CANDIDATE THESIS

Altered Molecular Profile in Thyroid Cancers From Patients Affected by the Three Mile Island Nuclear Accident

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Objectives/Hypothesis: In 1979, Three Mile Island (TMI) nuclear power plant experienced a partial meltdown with release of radioactive material. The effects of the accident on thyroid cancer (TC) in the surrounding population remain unclear. Radiation-induced TCs have a lower incidence of single nucleotide oncogenic driver mutations and higher incidence of gene fusions. We used next generation sequencing (NGS) to identify molecular signatures of radiation-induced TC in a cohort of TC patients residing near TMI during the time of the accident.

Study Design: Case series.

Methods: We identified 44 patients who developed papillary thyroid carcinoma between 1974 and 2014. Patients who developed TC between 1984 and 1996 were at risk for radiation-induced TC, patients who developed TC before 1984 or after 1996 were the control group. We used targeted NGS of paired tumor and normal tissue from each patient to identify single nucleotide oncogenic driver mutations. Oncogenic gene fusions were identified using quantitative reverse transcription polymerase chain reaction.

Results: We identified 15 patients in the at-risk group and 29 patients in the control group. $BRAF_{V600E}$ mutations were identified in 53% patients in the at-risk group and 83% patients in the control group. The proportion of patients with BRAF mutations in the at-risk group was significantly lower than predicted by the The Cancer Genome Atlas cohort. Gene fusion or somatic copy number alteration drivers were identified in 33% tumors in the at-risk group and 14% of tumors in the control group.

Conclusions: Findings were consistent with observations from other radiation-exposed populations. These data raise the possibility that radiation released from TMI may have altered the molecular profile of TC in the population surrounding TMI.

Key Words: Papillary thyroid cancer, radiation, Three Mile Island, next-generation sequencing.

Level of Evidence: 4

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INTRODUCTION

On the morning of March 28, 1979, a series of unlikely events led to a partial core meltdown in the #2 reactor of the Three Mile Island nuclear power plant (TMI). These events would ultimately lead to the worst civilian nuclear accident in United States history, culminating in the release of an estimated 2.4 to 13 million curies of radioactive material, mostly in the form of noble gases.[1,2] In addition, approximately 18.1 million curies of radioactive ^{131}I was produced during the accident, although most of this was contained within the reactor, with an estimated release of only 13 to 17 curies.[1] Despite assertions from the Nuclear Regulatory Commission and an independent Presidential Commission that the health effects of this release were likely to be minimal,[1,2] significant resources have been devoted to understanding the public health impact of the TMI meltdown.

One of the most intensely studied diseases following the TMI accident has been thyroid cancer. The most common form of thyroid cancer in the United States is papillary thyroid cancer (PTC),[3,4] for which radiation exposure is a known risk factor.[5,6] Several groups have conducted epidemiologic studies aimed at determining the effect of radiation released during the TMI accident on thyroid cancer in the surrounding population.[7–9] However, these efforts have produced variable results regarding the potential impact of the TMI accident on thyroid cancer incidence. Much of the variability associated with these studies is likely due to the relatively small size of the population surrounding the TMI plant relative to the large population required to detect

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statistically significant increases in cancer incidence following low-level radiation release, combined with a high degree of mobility in the local population. Therefore, we reasoned that novel approaches were required to identify potential effects of radiation released during the TMI accident on the local population.

To date, most studies of radiation-induced thyroid cancer have focused on populations with a history of significant radiation exposure, including childhood therapeutic radiation for cancer, childhood x-ray therapy for benign disease, survivors of the Chernobyl nuclear power plant meltdown, and survivors of the atomic bomb blasts in Hiroshima and Nagasaki.[10–16] Thyroid cancers in these radiation-exposed populations exhibit unique characteristics compared to populations of patients with sporadic thyroid cancers. Demographically, radiation exposure has the greatest impact on individuals exposed as children and produces thyroid cancer with a peak latency of 20 to 30 years after exposure.[15] Therefore, individuals with a history of radiation exposure typically develop thyroid cancer at a younger age than patients with sporadic thyroid cancer, for whom the median age at diagnosis is 51 years.[17]

In addition to demographic changes, distinct tumor genetic profiles have been observed in populations with radiation-induced thyroid cancers compared to patients with sporadic thyroid cancer. In general, thyroid cancers are characterized by a low somatic mutation rate, with the vast majority of cancers containing a single oncogenic driver mutation.[18] Among the 259 sporadic PTCs sequenced by The Cancer Genome Atlas (TCGA), single nucleotide point mutations including $BRAF_{V600E}$, $KRAS_{Q61R}$, $HRAS_{Q61R}$, and $NRAS_{Q61R}$ were identified in 200 (77%) cancers. Of the remaining 59 tumors, oncogenic gene fusions were identified in 41 (16%) tumors, oncogenic copy number alterations (CNA) drivers were present in 10 (4%), no driver was identified in seven (3%), and a rare $CHEK2_{N186S}$ driver was identified in one (0.4%) tumor.[18] By contrast, small series of patients with radiation-induced thyroid cancers have identified oncogenic gene fusion drivers in 23% to 84% of tumors, with a corresponding decrease in the frequency of oncogenic point mutations.[10,12,13,16,19–23] Furthermore, recent evidence suggests that CNAs may be identified with higher frequency in radiation-induced thyroid cancers compared to sporadic controls.[24] Although no one marker has been identified to specifically determine whether an individual tumor is radiation-induced, these data indicate an increased incidence of gene fusion or CNA thyroid cancer drivers, combined with a decreased prevalence of single nucleotide oncogenic driver mutations, is suggestive of population-level radiation exposure.

To identify molecular signatures of radiation-induced thyroid cancer in the population surrounding the TMI plant, we used a multifaceted molecular approach, including next-generation sequencing, to identify oncogenic driver mutations in a population of thyroid cancer patients who were life-long residents of the geographic area affected by the TMI accident. Strikingly, we identified an altered driver mutation profile in thyroid cancers that developed during the expected latency period following the incident compared to control patients from the same geographic area. Therefore, our results provide novel evidence for an impact of radiation released during the TMI partial core meltdown on thyroid cancer in the regional population.

MATERIALS AND METHODS

The institutional review board at PennState Health–Milton S. Hershey Medical Center approved this study.

Selection of Cases

Cases were identified from thyroid cancer patient records at the PennState Health–Milton S. Hersey Medical Center based on the availability of formalin-fixed paraffin-embedded (FFPE) tissue specimens at an off-site storage facility (Iron Mountain, Boyers, PA). All patients included in the study were born prior to the TMI accident on March 28, 1979. To account for geographic variations in thyroid cancer incidence and to control for potential differences in environmental exposures, all individuals included in the study resided in Pennsylvania during the time of the TMI accident, remained in Pennsylvania until the time of thyroid cancer development, and received surgical treatment for thyroid cancer at PennState Health–Milton S. Hershey Medical Center. We rigorously verified the birthplace and place of residence at the time of the TMI accident for each patient using data from a variety of sources including: genealogy software (ancestry.com), social security numbers, home and employment address records, patient charts, social networking websites (to determine residence during college or other educational activities), whitepages.com, intelius.com, spokeo.com, obituary pages, and military records.

To identify potential effects of the TMI accident on thyroid cancer biology, patients were divided into at-risk and control groups. We defined patients in the at-risk group to be those who developed thyroid cancer between 1984 and 1996, consistent with known latency periods of radiation-induced thyroid cancer,[15] and who lived in at-risk geographical areas at the time of the accident. At-risk regions were defined based on reported weather patterns at the time of the accident and subsequent epidemiological studies, and include Dauphin County, northern York County, eastern Cumberland County, western Lancaster County, and western Lebanon County (Fig. 1). Patients who developed thyroid cancer outside of the expected latency period were placed in the control group (Fig. 1A).

DNA and RNA Isolation

Experienced, subspecialized thyroid pathologists (H.C. and J.I.W.) identified tumor and adjacent normal thyroid tissue within FFPE blocks. For each patient, genomic DNA and total RNA were isolated from tumor and adjacent normal thyroid tissue using an AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To remove nucleic acid fragments <100 bp resulting from FFPE-induced damage, DNA and RNA samples were size-selected using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). DNA was quantified by quantitative polymerase chain reaction (PCR) using TaqMan RNase P Detection Reagents (FAM) (Life Technologies, Austin, TX) and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) on a QuantStudio 12K Flex Real Time PCR Station with 12K Flex software (Applied Biosystems) according to the manufacturer's instructions for Ion Ampliseq sample quantification. RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA).

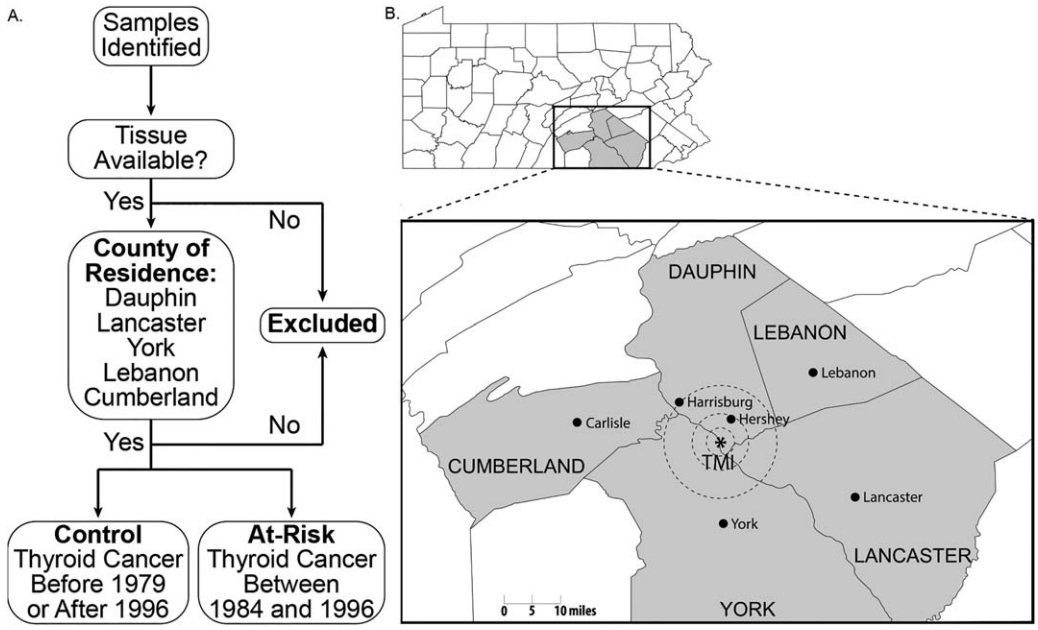

Fig. 1. Inclusion criteria. (A) A flow diagram depicting the criteria used to identify patients for the study and to stratify patients into at risk and control groups. (B) A map of the area surrounding the Three Mile Island nuclear power plant (asterisk). Patients were identified from counties shaded in grey. Dashed circles indicate 2.5-, 5-, and 10-mile radii from the plant.

Ion AmpliSeq Comprehensive Cancer Panel Library Sequencing

For each sample, 10 ng of isolated DNA was mixed with each Comprehensive Cancer Panel primer pool (Thermo Fisher Scientific) and amplified using an Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) on a Veriti 96-well Thermal Cycler (Applied Biosystems) according to the manufacturer's instructions. Individual libraries were barcoded using Ion Xpress Barcode Adapters (Thermo Fisher Scientific), purified using Agencourt AMPure XP beads, and quantified using the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific) on a QuantStudio 12K Flex Real Time PCR Station.

Libraries were templated in sets of four matched tumor/normal pairs using Ion PI Hi-Q OT2 Reagents 200 (Thermo Fisher Scientific) kit on an Ion OneTouch 2 System (Thermo Fisher Scientific) according to the manufacturer's instructions. Templated libraries were quantitated using the Ion Sphere Quality Control Kit (Thermo Fisher Scientific) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Libraries passing quality control were enriched using Dynabeads MyOne Streptavadin C1 Beads (Thermo Fisher Scientific) on an Ion OneTouch ES (Thermo Fisher Scientific). Following enrichment, the libraries were loaded onto an Ion PI v3 Chip (Thermo Fisher Scientific) and sequenced on the Ion Proton system (Thermo Fisher Scientific) utilizing the Ion PI Hi-Q Sequencing 200 Kit (Thermo Fisher Scientific) with 520 flows according to the manufacturer's instructions. Eight libraries composed of four tumor-normal pairs were sequenced per chip, resulting in an average depth of coverage $>250\times$ per sample. Importantly, all samples were subjected to two independent rounds of sequencing to account for FFPE-induced DNA damage.[25]

Sequencing Data Analysis

Ampliseq next generation sequencing data were processed on the Ion Proton and Ion Torrent T620 server (Thermo Fisher Scientific) using the manufacturer's Ion Torrent data analysis pipeline. Briefly, raw base calls are determined relative to background signal and assigned a quality value (QV). Raw reads are then subjected to quality filtering to remove low-quality reads, barcode classification, and trimming of low quality 3′ bases and adapters. After filtering and trimming, the remaining reads aligned to the hg19 reference using the Torrent Mapping Alignment Program, which includes a proprietary novel alignment algorithm (super-maximal exact matching) designed to address the specific features of Ion Torrent data, including variable read length and higher incidences of random insertions and deletions due to miscalled homopolymers.

Somatic nucleotide variant calling was conducted on the Ion Reporter Cloud system (https://ionreporter.thermofisher.com/ir; Thermo Fisher Scientific) using the proprietary Ampliseq Tumor/Normal workflow. This pipeline consists of a combination of algorithms aimed at detecting single nucleotide variants, insertions, and deletions in Ion Torrent sequencing data. Sequencing reads were filtered to remove low QV reads and bases, low coverage depth ($<10\times$), and strand bias. Minor alleles were called using the frequentist algorithm, which determines whether the number of observed alternative alleles is higher than those estimated by chance, and incorporates a correction estimate for variants detected near homopolymers due to increased false positives.

Indels were called using a modification of the Genome Analysis ToolKit UnifiedGenotyper tool within the Ampliseq Tumor/Normal workflow that identifies candidate indels present at $10\times$ coverage or higher. This algorithm accounts for the homopolymer-induced indels intrinsic to Ion Torrent sequencing data.[26] Large indels are identified by scanning Binary Alignment/Map (BAM) files for >10 bp of nonaligned sequences. These partial sequences are then reassembled from partially aligned reads and called as indel if successfully aligned to the reference. Finally, all called indels were filtered based on score, frequency, and strand bias. Somatic copy number variants were called using the ONCOCNV algorithm for amplicon-based sequencing data.[27]

Reverse Transcription PCR for RET/PTC 1 and RET/PTC 3 Chromosome Rearrangements

To identify RET/PTC1 gene fusion transcripts, total cellular RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. A portion of cDNA was amplified using primers for RET/PTC1 as described previously[28] with OneTaq DNA polymerase (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions. Specific PCR products were identified by 2% agarose gel electrophoresis. Bands matching the expected fragment size were gel purified using a QiaQuick Gel Extraction Kit (Qiagen) and confirmed by Sanger sequencing (Genewiz, South Plainfield, NJ). To identify RET/PTC3 translocations, quantitative reverse transcription PCR was conducted using a previously described primer set.[28] Quantitative PCR was carried out using Gene Expression Master Mix (Thermo Fisher Scientific) with cycling conditions set at the manufacturer recommendations on a QuantStudio 12K Flex Real Time PCR Station.

Statistical Testing

The statistical significance of the association between BRAF point mutation status (mutant vs. wild type) and risk group (at risk vs. control) was assessed using a one-sided Fisher exact test in which the alternative hypothesis was that BRAF point mutations are less frequent in the at-risk group. A one-sided test was chosen because previous studies suggest that BRAF point mutations are less common in PTCs associated with radiation exposure.[10,12,13,16,19–23] In an effort to assess the statistical significance of the frequency of BRAF point mutations in the exposed group, we employed a resampling-based approach using data from the TCGA cohort, the largest PTC study to date.[18] Briefly, an empirical distribution of BRAF point mutation frequencies was created based on 1,000,000 random samples chosen from the n = 259 classical PTCs in the TCGA study.[18] Random sampling was conducted twice, with selections equal to the size of the at-risk group (n = 15) or the control group (n = 29), and a two-sided *P* value for the observed frequency of BRAF mutations in the exposed group was computed using the empirical distribution. Wilcoxon rank sum and Fisher exact tests were implemented using the R environment for statistical computing v3.2.2.[29] Heat maps and graphs were generated using the NMF[30] and ggplot2[31] packages for R v3.2.2.

RESULTS

Patient Characteristics

We identified 44 patients who were treated for thyroid cancer at our institution between 1974 and 2014 and who met all inclusion criteria (Fig. 1A, Table I). Fifteen patients developed thyroid cancer between 1984 and 1996, and were therefore classified as at-risk for radiation-induced disease (Table I). By contrast, 29 patients developed thyroid cancer before 1984 or after 1996, and were therefore classified as the control group given their lower risk of having radiation-induced disease (Table I). The male:female ratio and county of residence distributions were similar between the at-risk and control groups. However, patients in the at-risk group developed thyroid cancer an average of 10.8 years earlier than observed in the control group (Table I, Fig. 2A) ($P = .05$). Per our inclusion criteria, all thyroid cancers in our cohort exhibited classic papillary architecture as determined by two subspecialized thyroid pathologists (H.C. and J.I.W.) (data not shown).

TABLE I.
Demographic Characteristics of Patients Meeting Inclusion Criteria

	At Risk, n = 15	Control, n = 29	*P* Value
Sex			1.00*
Male	4	7	
Female	11	22	
Mean age at exposure ± SD, yr	28.1 ± 18.0	24 ± 15.4	.41†
Mean age diagnosis ± SD, yr	36.9 ± 15.5	47.7 ± 17.1	.05†
County			.52*
Dauphin	7	13	
Cumberland	2	9	
Lancaster	1	1	
Lebanon	5	5	
York	0	1	

*Fisher exact test
†Wilcoxon rank sum test
SD = standard deviation.

Identification of Somatic Point Mutations

To identify oncogenic drivers in the at-risk and control populations, we conducted next-generation sequencing of > 400 cancer-associated genes to a depth of >200 × to > 1,000 × in paired tumor and normal tissue from each patient (Fig. 2A). Somatic point mutations were detected in 32 of 44 patients, with $BRAF_{V600E}$ representing the only single nucleotide oncogenic driver mutation identified in our cohort (Fig. 2A). Consistent with previous reports,[18] our cohort had a low frequency of somatic single nucleotide mutations, corresponding to ~0.5 mutations per megabase (Fig. 2A) (data not shown).

To identify potential effects of ionizing radiation on the mutational signature of thyroid cancers in our cohort, we tested whether the frequency of $BRAF_{V600E}$ mutations was lower in the at-risk group than the control group. Only eight of 15 (53%) tumors in the at-risk group were positive for $BRAF_{V600E}$, compared to 24 of 29 (83%) tumors in the control group (Fig. 2B). This difference was statistically significant ($P = .04$), indicating that the proportion of $BRAF_{V600E}$-positive tumors is lower in the at-risk group compared to the control group. Patient age at diagnosis was not independently associated with $BRAF_{V600E}$ mutation status (data not shown).

Recently, TCGA published the comprehensive genetic analysis of approximately 400 thyroid cancers obtained from diverse geographic regions across the United States.[18] To test whether a small sample size skewed the distribution of oncogenic driver mutations in our cohort, we used a Monte Carlo approach to iteratively sample the 259 classic PTCs included in the TCGA database with a sample size of 15 (corresponding to the affected group) or 29 (corresponding to the control group). The sampling process was repeated 1 million

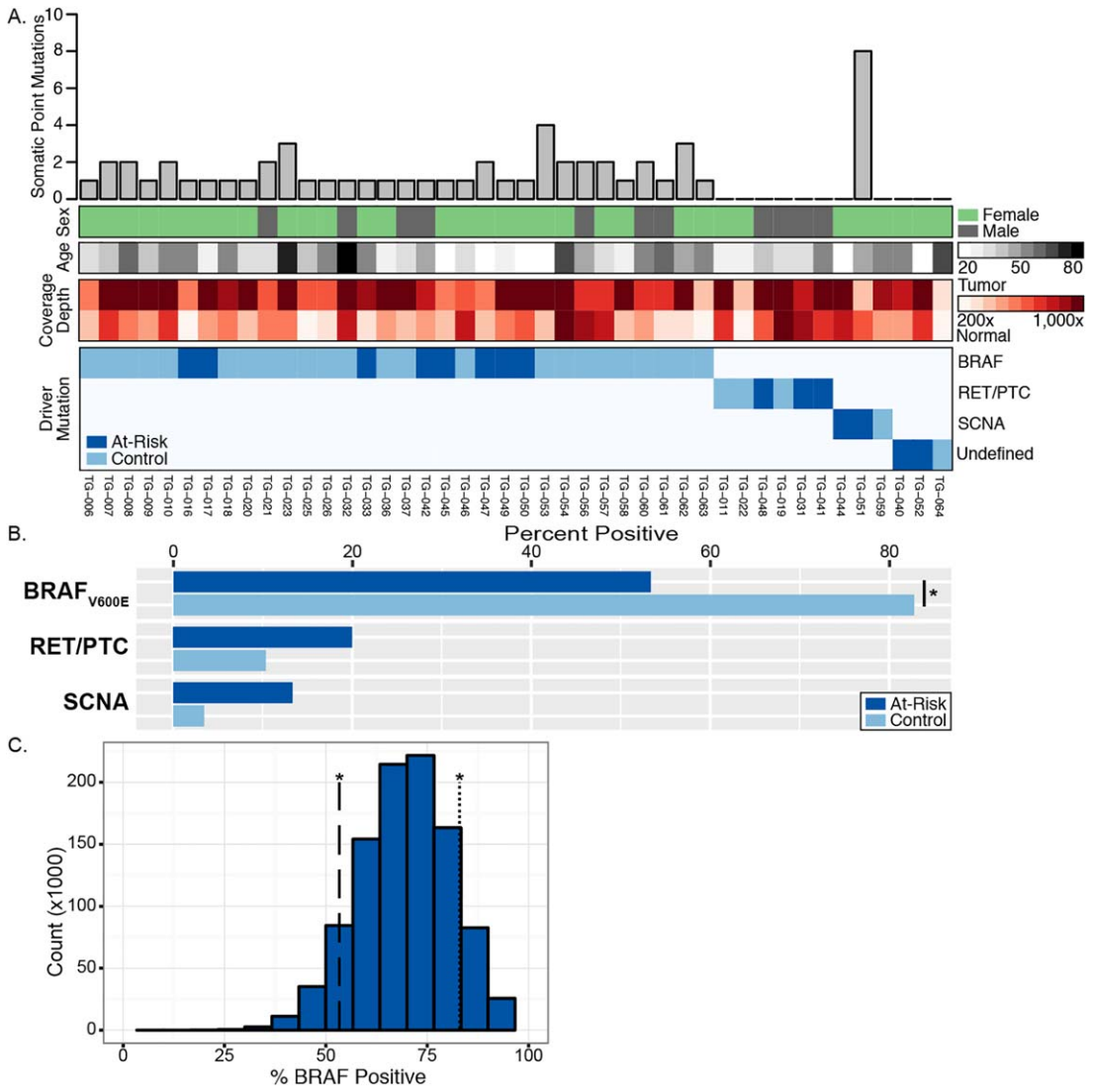

Fig. 2. Demographic and molecular characteristics of thyroid cancer patients included in the study. (A) The bar graph indicates the number of somatic mutations identified in each tumor by next-generation sequencing. Heat maps indicate patient sex, age at diagnosis, sequencing coverage depth, and oncogenic driver mutation status. (B) The bar graphs depict the proportion of tumors with BRAF, gene fusion, and copy number alterations driver mutations in the at-risk and control groups. (C) Histogram displaying the frequency distribution of BRAF-positive tumors generated by sampling The Cancer Genome Atlas dataset 1,000,000 times with a selection size of 15, corresponding to the size of the at-risk group. The dashed line indicates the proportion of BRAF-positive tumors in the at-risk group, whereas the dotted line represents the proportion of BRAF-positive tumors in the control group from this study.

times, and the percentage of tumors with single nucleotide oncogenic driver mutations was determined for each iteration (Fig. 2C). Using this approach, we found that the frequency of BRAF mutations in the at-risk population was significantly lower than would be expected by randomly selecting a group of 15 tumors from the TCGA cohort (Fig. 2C) ($P = .042$). By contrast, the percentage of tumors with BRAF mutations in the control group was significantly larger than expected (Fig. 2C) ($P = .023$). Importantly, of the 259 classic PTCs in the TCGA cohort, only nine patients reported a history of radiation exposure,[18] indicating that the TCGA cohort represents the expected mutational frequency for patients with sporadic thyroid cancer.

Identification of Additional Oncogenic Driver Mutations

To identify oncogenic driver mutations in the 12 tumors in our cohort without identifiable oncogenic point mutations, we assayed for gene fusions involving the RET protooncogene and CCDC6 (RET/PTC1) or NCOA4 (RET/PTC3), which are the most common gene fusions in PTC and are over-represented in radiation-induced thyroid cancer.[19–21] In total, reverse transcription PCR for RET/PTC1 or RET/PTC3 gene fusions were identified in three tumors in the at-risk group (20%) and three tumors in the control group (10%) (Fig. 2A,B). In the at-risk group, one tumor was positive for RET/PTC1 and two tumors were positive for RET/PTC3, whereas in the

TABLE II.
Copy Number Alteration Drivers Identified in Tumors Without Single Nucleotide or Gene Fusion Driver Mutations

Patient	Affected Region	Alteration	CN	Affected Genes
TG044	Chr2:128046282 -216272375	Pot. loss	1.5	*ERCC3, LRP1B, ACVR2A, NFE2L2, PMS1, SF3B1, CREB1, IDH1, ERBB4, FN1*
	Chr6:160452062 - 167275553	Pot. gain	2.5	*IGF2R,RPS6KA2*
	Chr8: 71074909 - 119122492	Pot. loss	1.5	*NCOA2, NBN, RUNX1T1, UBR5, CSMD3, EXT1*
	Chr12: 25368376 - 46244408	Pot. loss	1.5	*KRAS, ADAMTS20, ARID2*
	Chr16: 2098562 – 89882283	Pot. gain	2.5	*TSC2, CREBBP, SOCS1, ERCC4, MYH11, PALB2, IL21R, CYLD, MMP2, CDH11, CDH5, CDH1, MAF, FANCA*
	Chr17: 5405081 – 8110704	Pot. gain	2.5	*NLRP1, TP53, PER1, AURKB*
	Chr17: 75475041 – 78367208	Pot. gain	2.5	*SEPT9, BIRC5, RNF213*
	Chr18: 22669326 – 60985847	Pot. loss	1.5	*ZNF521, CDH2, SMAD2, MBD1, SMAD4, DCC, MALT1, CDH20, BCL2*
	Chr19: 1206845 - 57746677	Pot. gain	2.5	*STK11, TCF3, GNA11, FZR1, MAP2K2, KEAP1, SMARCA4, JAK3, PIK3R2, CRTC1, CCNE1, CEBPA, AKT2, AXL, CD79A, CIC, BCL3, MARK4, ERCC2, ERCC1, PPP2R1A, AURKC*
	Chr22:21272220 - 42526658	Pot. gain	2.5	*CRKL, MAPK1, BCR, SMARCB1, MN1, CHEK2, NF2, TIMP3, MYH9, PDGFB, EP300, CYP2D6*
TG051	Chr2:134520981 - 142402899	Pot. loss	1.5	*LRP1B*
	Chr2:213196361 - 219542398	Pot. gain	2.5	*ERBB4, FN1, STK36*
	Chr2:219542398 - 234681120	Pot. loss	1.5	*STK36, PAX3, UGT1A1*
	Chr3:80202083 - 138374621	Pot. loss	1.5	*EPHA3, GATA2, EPHB1, PIK3CB*
	Chr4:40996673 – 62936076	Pot. loss	1.5	*PHOX2B, PDGFRA, KIT, KDR, LPHN3*
	Chr4:62936076 - 153332857	Pot. gain	2.5	*LPHN3, AFF1, NFKB1, TET2, IL2, FBXW7*
	Chr8:108305134 - 118848494	Pot. loss	1.5	*CSMD3, EXT1*
	Chr8:118848494 - 128753145	Gain	3	*EXT1, MYC*
	Chr14: 51281011 - 62213091	Gain	3	*NIN, HIF1A*
	Chr19:1206845 - 57746677	Pot. gain	2.5	*STK11, TCF3, GNA11, FZR1, MAP2K2, KEAP1, SMARCA4, JAK3, PIK3R2, CRTC1, CCNE1, CEBPA, AKT2, AXL, CD79A, CIC, BCL3, MARK4, ERCC2, ERCC1, PPP2R1A, AURKC*
	Chr22:21272220 - 42526658	Pot. gain	2.5	*CRKL, MAPK1, BCR, SMARCB1, MN1, CHEK2, NF2, TIMP3, MYH9, PDGFB, EP300, CYP2D6*
TG059	Chr4:1800963 - 1979297	Pot. loss	1.5	*FGFR3, WHSC1*
	Chr8:119122370 - 145742416	Pot. loss	1.5	*EXT1, MYC, RECQL4*
	Chr9:135889942 - 139438447	Pot. loss	1.5	*RALGDS, BRD3, NOTCH1*
	Chr11:47246420 - 94166025	Pot. loss	1.5	*DDB2, MEN1, CCND1, NUMA1, MRE11A*
	Chr16:2098562 - 3817809	Pot. loss	1.5	*TSC2, CREBBP*
	Chr16:68845845 - 89882283	Pot. loss	1.5	*CDH1, MAF, FANCA*
	Chr19:1206845 - 57746677	Pot. loss	1.5	*STK11, TCF3, GNA11, FZR1, MAP2K2, KEAP1, SMARCA4, JAK3, PIK3R2, CRTC1, CCNE1, CEBPA, AKT2, AXL, CD79A, CIC, BCL3, MARK4, ERCC2, ERCC1, PPP2R1A, AURKC*
	Chr20:39802968 - 54957288	Pot. gain	2.5	*PLCG1, PTPRT, AURKA*

CN = copy number; Pot. loss = potential copy loss (CN = 1.5); Pot. gain = potential copy gain (CN = 2.5); Gain = copy gain (CN $\geq$ 3).

control group, two tumors were positive for RET/PTC1 and one tumor was positive for RET/PTC3 (data not shown). Importantly, Sanger sequencing or quantitative PCR was used to confirm the identity of all gene fusions.

In addition to gene fusions, somatic copy number alterations (SCNAs) may play an important role in the oncogenesis of sporadic and radiation-induced thyroid cancer.[18,24,32] To identify copy number alterations in our amplicon sequencing data, we used the ONCOCNV algorithm, which achieves a degree of precision similar to more traditional comparative genomic hybridization arrays.[27] Using this approach, we identified SCNA driver mutations in two tumors from the at-risk group (13%) and one tumor from the control group (3%) in which we identified no other oncogenic driver mutations. We detected similar copy number gains in chromosomes 19 and 22 for the two tumors in the at-risk group, TG044 and TG051, which were not identified in the tumor from the control group (Table II). Furthermore, in tumor TG051 we identified a focal amplification of the *Myc* gene (Table II), which was not identified in the TCGA cohort.[18] Overall, 11 and 10 distinct alterations in copy number

were identified in the two tumors from the at-risk group, whereas eight distinct copy number alterations were identified in the tumor from the control group (Table II).

DISCUSSION

Since the TMI accident in 1979, the effect of the released radiation on the local population has remained controversial. This study represents the first effort to identify molecular signatures of radiation-induced thyroid cancer in the population surrounding TMI. Among patients with sporadic thyroid cancer, point mutations in the BRAF or RAS family of tyrosine kinases are the most commonly identified oncogenic drivers.[18,33] Driver mutations in BRAF, HRAS, KRAS, or NRAS were identified in 77% of the 259 classical PTCs sequenced by the TCGA.[18] Here, we used a targeted next-generation sequencing approach to identify oncogenic driver mutations among life-long Pennsylvanians residing near the TMI nuclear power plant. Among control patients, who were at lower risk for radiation-induced thyroid cancer stemming from the TMI accident, $BRAF_{V600E}$ mutations were identified in nearly 83% of patients. By contrast, $BRAF_{V600E}$ mutations were identified in only 53% of patients residing in the same geographic area who developed thyroid cancer between 5 and 17 years following the accident. Lower incidences of single nucleotide oncogenic driver mutations also have been observed in thyroid cancers among survivors of the atomic bomb in Hiroshima and the Chernobyl reactor meltdown,[23,34] suggesting that radiation-induced DNA damage may have contributed to the development of thyroid cancers in the area surrounding TMI reactor.

Although $BRAF_{V600E}$ was the only oncogenic driver mutation identified in our cohort, likely as a result of our selection of only PTCs for inclusion, it is important to note that the sequencing panel used here included >400 cancer-associated genes. The number of genes covered by this panel is important because PTCs generally are associated with only a limited number of oncogenic driver mutations. In the group of classical PTCs sequenced by the TCGA, $BRAF_{V600E}$ and RAS-family mutations were identified in 78% of all tumors. For all tumors in our cohort, BRAF and all RAS-family proteins were covered at high depth, making it extremely unlikely that we would miss an oncogenic driver mutation in one of these genes. Within the TCGA cohort, oncogenic driver mutations were identified in only one additional gene, *EIF1AX*, which was not covered by our sequencing panel. However, only two of the 259 classic papillary thyroid cancers sequenced by the TCGA were positive for this mutation. Therefore, although it is possible that the observed decrease in oncogenic point mutations observed in our cohort results from mutations in genes not covered in our panel, this possibility is unlikely.

In support of our finding that the incidence of single nucleotide driver mutations was decreased in the at-risk group, we identified alternative driver mutations in five of the remaining seven tumors in the at-risk group and four of the five remaining tumors in the control group. A majority (six) of these alternative driver mutations represented RET/PTC translocations, which have a higher frequency in thyroid cancer patients with a history of significant radiation exposure and form in response to radiation exposure in vitro.[10,14,21,35] Interestingly, the three patients in the control group with RET/PTC gene fusions were ages 1, 4, and 8 years at the time of exposure (Fig. 2A) (data not shown). Children are at higher risk for radiation-induced thyroid cancer,[36] raising the possibility that these patients represent cases of radiation-induced thyroid cancer with a delayed presentation. However, further data are required to definitively prove this possibility.

Among the six tumors without definitive single nucleotide or gene fusion driver mutations, we identified three tumors with CNA drivers, two in the at-risk group and one in the control group (Fig. 2A,B). Like intrachromosomal translocations, CNAs are also over-represented in radiation-induced thyroid cancer.[24] Because the vast majority of thyroid cancers have only one oncogenic driver mutation, these data provide further evidence that the mutational profile of thyroid cancers in the at-risk group represents a true variation from the expected mutation profile.

Our finding that some thyroid cancer samples lack detectable oncogenic driver mutations is neither unexpected nor unprecedented. In the TCGA cohort, which included a more extensive search for oncogenic driver mutations, seven of the 259 classic papillary thyroid cancers (2.7%) had no detectable oncogenic drivers.[18] Additionally, translocations other than RET/PTC1 and RET/PTC3 have been reported in thyroid cancer patients with a history of radiation exposure.[13,37] Given the technical limitations imposed by obtaining intact nucleic acids, particularly RNA, from nearly 50-year-old formalin-fixed tumor samples, we were not able to carry out an exhaustive search for alternative translocations. Therefore, it is possible that true oncogenic drivers are present in the three tumors for which we were not able to identify driver mutations. However, it is important to note that these tumors are unlikely to harbor single nucleotide driver mutations given our extensive sequencing analysis.

This study stands in contrast to several previous reports describing the radiation risks associated with the TMI accident. Government reports following the accident concluded that the radiation released during the accident posed little risk to human health.[1,2] Furthermore, short-term studies conducted in the years following the accident demonstrated little to no increased risk of developing cancer or cancer-related mortality in the population surrounding the plant.[38–40] However, detecting population-level effects of radioactive release remains challenging. Primarily, large populations are required to detect significant increases in cancer mortality following low-dose radiation exposure. A population size of ~500,000 individuals with lifetime follow-up would be required to detect a significant increase in cancer deaths assuming the 25-mrem likely exposure for individuals living within 5 miles of the plant.[39,41] However, the TMI cohort, a registry of individuals living

within 5 miles of the plant that was compiled immediately following the accident, contained only 32,563 residents and was subject to high levels of migration, with 3,790 people moving out of the area within the first year.[40,42] Accordingly, the failure of previous studies to detect significant increases in cancer risk following the TMI accident might be due to the small size of the population and the high degree of population mobility following the accident.

There are several possible reasons why our study identified potential effects of the TMI accident on thyroid cancer when other studies have failed to detect a significant association. Primarily, we used an extensive vetting process to ensure that patients included in our study were present in at-risk counties at the time of the accident and to confirm, to the greatest extent possible, that patients resided in affected areas for their entire lives. Therefore, our study represents a static population, which increased our ability to detect radiation-induced cancers. In addition, our approach relies on molecular profiling to detect radiation-induced cancers among a population of thyroid cancer patients rather than attempting to detect an increase in thyroid cancer incidence. This distinction is important because thyroid cancer remains a relatively rare disease in the general population.[17] Accordingly, small annual variations in thyroid cancer diagnosis produce a high degree of variability in disease incidence, making it difficult to detect significant changes in disease incidence. By dividing patients into at-risk and control groups based on the expected latency of radiation-induced thyroid cancer and focusing on the detection of radiation-associated mutations, we were able to eliminate much of the variability associated with population-based studies.

It is important to recognize several limitations associated with this study. The first is the relatively small sample size of our study population. This results from both the age of the tissue samples included in the study, as well as the extensive vetting process to ensure that all patients included in the study resided within potentially affected areas during the entirety of the study period. Despite exhaustive searches, no further samples meeting inclusion criteria could be identified. The age and storage conditions of the identified samples also introduced technical limitations to this study. For example, formalin fixation produces characteristic patterns of random DNA mutations,[43] which required us to conduct two independent sequencing runs for each sample to identify true mutations amidst the high background of formalin-induced mutations. Furthermore, the age of many samples resulted in significant RNA degradation, which limited our ability to identify gene fusions by reverse transcription PCR. Despite these limitations, the depth of sequencing coverage achieved for all samples allowed us to identify single nucleotide oncogenic driver mutations confidently in all tumors in which such mutations were present.

Second, although our data show an altered mutational profile for thyroid cancers that developed during the expected latency period following the TMI accident, we cannot definitively establish that this effect is due to the TMI accident. For example, we found that patients in the at-risk group were younger at diagnosis than patients in the control group. Thyroid cancer in young patients is associated with a higher frequency of gene fusion driver mutations, although this association has been detected primarily in the context of pediatric thyroid cancer.[44] Patient age at diagnosis was not independently associated with $BRAF_{V600E}$ mutation status, and no pediatric patients were included in our study, indicating that age per se is unlikely to have been a strong contributor to the altered mutational profile observed in the at-risk group.

Radon gas is also a prevalent source of environmental radiation exposure, particularly in central Pennsylvania, which has led others to postulate that radon exposure may play a role in thyroid tumorigenesis. However, recent evidence has shown no geospatial relationship between radon exposure and thyroid cancer,[45] and the control population actually had a significantly higher rate of single nucleotide oncogenic driver mutations than would be expected based on TCGA data (Fig. 2C). Therefore, these data suggest a radiation source other than environmental radon exposure contributed to the development of thyroid cancer in the at-risk population. However, it is important to note that no single molecular test can determine specifically whether an individual tumor was induced by radiation exposure.

CONCLUSION

Our data show a decrease in the frequency of single nucleotide oncogenic driver mutations in a population of thyroid cancer patients who may have been affected by radiation released during the TMI nuclear accident. This result is consistent with observations in other populations of thyroid cancer patients with significant radiation exposure, suggesting that radiation released during the TMI accident may have contributed to the development of thyroid cancer in some patients. Further research is required to definitively determine the role of the TMI accident on thyroid cancer in the surrounding population.

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