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Role of *CDKN2C* Copy Number in Sporadic Medullary Thyroid Carcinoma

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Background: The cyclin-dependent-kinase inhibitors (CDKN)/retinoblastoma (RB1) pathway has been implicated as having a role in medullary thyroid carcinoma (MTC) tumorigenesis. *CDKN2C* loss has been associated with *RET*-mediated MTC in humans but with minimal phenotypic correlation provided. The objective of this study was to evaluate the association between tumor *RET* mutation status, *CDKN2C* loss, and aggressiveness of MTC in a cohort of patients with sporadic disease.
Methods: Tumors from patients with sporadic MTC treated at a single institution were evaluated for somatic RET^{M918T} mutation and *CDKN2C* copy number loss. These variables were compared to patient demographics, pathology detail, clinical course, and disease-specific and overall survival.
Results: Sixty-two MTC cases with an initial surgery date ranging from 1983 to 2009 met the inclusion criteria, of whom 36 (58%) were male. The median age at initial surgery was 53 years (range 22–81 years). The median tumor size was 30 mm (range 6–145 mm) with 29 (57%) possessing extrathyroidal extension. Nodal and/or distant metastasis at presentation was found in 47/60 (78%) and 12/61 (20%) patients, respectively. Median follow-up time was 10.5 years (range 1.1–27.8 years) for the censored observations. The presence of *CDKN2C* loss was associated with worse M stage and overall AJCC stage. Median overall survival of patients with versus without *CDKN2C* loss was 4.14 [confidence interval (CI) 1.93–NA] versus 18.27 [CI 17.24–NA] years ($p < 0.0001$). Median overall survival of patients with a combined somatic RET^{M918T} mutation and *CDKN2C* loss versus no somatic RET^{M918T} mutation and *CDKN2C* loss versus somatic RET^{M918T} mutation and *CDKN2C* 2N versus no somatic RET^{M918T} mutation and *CDKN2C* 2N was 2.38 [CI 1.67–NA] years versus 10.81 [CI 2.46–NA] versus 17.24 [CI 9.82–NA] versus not reached [CI 13.46–NA] years ($p < 0.0001$).
Conclusions: The detection of somatic *CDKN2C* loss is associated with the presence of distant metastasis at presentation as well decreased overall survival, a relationship enhanced by concomitant RET^{M918T} mutation. Further defining the genes involved in the progression of metastatic MTC will be an important step toward identifying pathways of disease progression and new therapeutic targets.

Keywords: medullary thyroid carcinoma, *CDKN2C*, *RET*, Rb pathway, haploinsufficiency

Introduction

Medullary thyroid carcinoma (MTC) derives from the calcitonin-producing parafollicular cells, and accounts for approximately 1200 new cancer diagnoses annually (1,2). While hereditary forms of MTC associated with Multiple Endocrine Neoplasia Type 2 (MEN2) can be successfully treated with prophylactic surgery, for sporadic presentation, patient outcomes are highly dependent on tumor stage. Without systemic treatment, five-year survival is <50% for individuals presenting with stage 3 or 4 disease (3). In recent years, there has been a paradigm shift in the treatment of advanced MTC, with the introduction of molecular targeted therapies. Inhibiting the action of key pathogenic tyrosine kinase, *RET*, has led to the Food and Drug Administration approval of vandetanib and cabozantanib for the treatment of MTC (4–6). These events represent an exciting development in the treatment of a cancer that has previously had few systemic options for patients with advanced disease. Unfortunately, these agents have not been curative, and the

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adverse effects often require mitigating therapies, leaving room for improvement (7–9).

While *RET* mutations, specifically RET^{M918T} alterations, have been identified as predominant driver pathways in sporadic MTC, these isolated defects do not explain the majority of cases, representing a knowledge gap in tumorigenesis. This primary target of systemic treatments accounts for only about 40% of MTC cases (10,11). Activating mutations in *RAS* have recently risen as a second driver of MTC in 10–15% of cases, and are not predicted to be directly impacted by therapies targeting *RET* (12,13). Thus, there is a clear need to define patient-specific mutations in order to personalize therapies better. In considering targets beyond *RET* and *RAS*, members of the cyclin-dependent-kinase inhibitors (CDKN)/retinoblastoma (RB1) tumor suppressor pathway have been implicated as having a role in MTC tumorigenesis (14–17). Universally, the pathway plays a critical role in cell cycle checkpoint regulation; alterations in one or more of various components may propagate aberrant cell proliferation and development of cancer. The members of the INK4/CDKN2 family (CDKN2A [p15], CDKN2B [p16], CDKN2C [p18], and CDKN2D [p19]) are cyclin-dependent kinase inhibitors that block the progression of the cell cycle by interacting with CDK4 or CDK6 to prevent activation of the Cyclin D-CDK4/6 complex (Fig. 1). The aberrant loss of CDKNs lead to unrestrained phosphorylation of RB and unregulated progression through the S phase of the cell cycle, and has been associated with the development of numerous cancers (18–21).

A role for CDKNs in MTC in humans is supported by two observations: (i) frequent loss (38%) of the 1p32 chromosomal region containing *CDKN2C* in sporadic MTC tumors examined by array CGH (22,23), and (ii) the finding of *CDKN2C* somatic mutations in 8.5% of studied samples (10,11). Haploinsufficiency occurs in a diploid organism when loss of gene function causes a phenotype, typically though mutation or copy number loss (24). Reduction of CDKN2C function by means of haploinsufficiency has a dose-dependent effect on tumorigenesis when combined with other oncogenic factors (25,26), and has been associated with *RET*-mediated MTC in humans (15,22). Additionally, loss of a single gene copy is sufficient to cause MTC in mice with the disease course accelerated by a concomitant *RET* mutation (14). Such alterations are suggested to impede *CDKN2C* function, implicating it as a halpoinsufficient tumor suppressor gene in malignancies including human MTC (27).

These findings provide the basis for our hypothesis that alterations within the CDKN2/RB1 pathway contribute to the development and progression of MTC in humans. The objective of this study was to evaluate the association between *RET* mutation status, *CDKN2C* halploinsufficiency through copy number loss, and aggressiveness of MTC in a cohort of patients with sporadic disease. If such an association exists between aberrations in cell cycle regulators and biological behavior in MTC, this pathway may be a viable target for MTC therapy, as targeted therapies that function through direct CDK inhibition are being developed across multiple human cancers.

Materials and Methods

MTC patients and clinical data

All cases were derived from patients who were treated at The University of Texas MD Anderson Cancer Center. A total of 62 sporadic MTC cases were included in this single-center study for which approval from the Institutional Review Board was obtained. Inclusion criteria for cases were: (i) having available primary tumor; (ii) known germline *RET* negative status (33 had germline testing that included exons 10, 11, 13–16; 29 had fewer exons examined appropriate to the era in which they were tested, but all had exon 16 tested); and (iii) availability of corresponding clinical data. Clinical

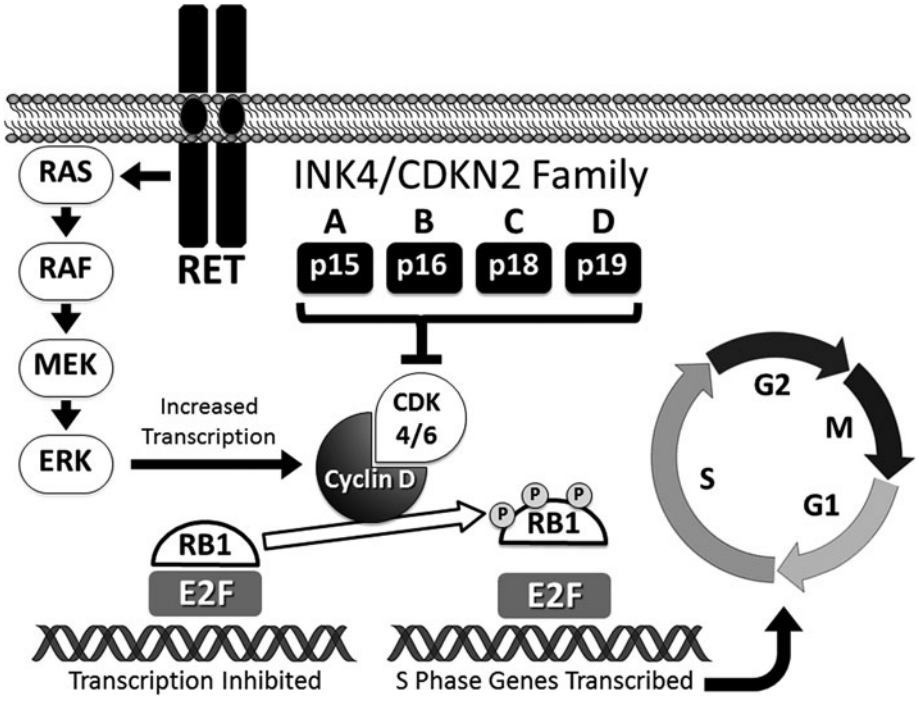

FIG. 1. Molecular pathways associated with thyroid cancer. Mutations in the *RET*/*MAPK* signaling pathway are known to drive tumorigenesis. This activation causes the enhanced progression of Cyclin D, which interacts with CDK4/6 to phosphorylate Rb. pRb is required for cell cycle progression. The members of the INK4/CDKN2 family (CDKN2A [p15], CDKN2B [p16], CDKN2C [p18], and CDKN2D [p19]) are cyclin-dependent kinase inhibitors that block the progression of the cell cycle by interacting with CDK4 or CDK6 to prevent activation of the Cyclin D-CDK4/6 complex.

data were retrieved from a prospectively maintained database within the Department of Surgical Oncology at the University of Texas MD Anderson Cancer Center. All pathology slides were evaluated by one of four dedicated head and neck pathologists at the institution. Pathology variables included tumor size of greatest dimension and presence of extrathyroidal extension. Invasion of adjacent structures was determined from combination of surgeon's report at time of operation and pathology record. TNM staging was based on 7th Edition AJCC criteria (28). Nodal status was assigned as NX if no nodal material was removed, and MX was assigned if no staging workup was performed when a preoperative calcitonin was >400 within six months of the diagnosis. Locoregional recurrence was defined as biopsy-proven disease within the neck more than six months after the initial surgery. Date of diagnosis was defined as date of initial surgery. Disease status was censored at last evaluation or time of death. Disease-free status was defined as no biopsy-proven neck disease and no radiographic evidence of distant disease; the latter required negative chest and abdomen computed tomography or magnetic resonance imaging (MRI) and bone scans, or spine MRI if the calcitonin levels were >400 pg/mL. Deaths were further categorized as due to MTC, other cause, and unknown cause. The last category included patients that all had advanced MTC at the time of their death, but the death could not be definitively linked to their disease. Disease-specific survival (DSS) was calculated from the date of surgery until death due to either MTC or unknown causes. Overall survival (OS) was measured from the date of diagnosis until death from any cause.

DNA isolation

Paired tumor/normal thyroid formalin-fixed, paraffin-embedded (FFPE) tissue was obtained from 47 MTC patients. An additional 11 FFPE tumor samples without paired normal tissue were also included in this study. A serial hematoxylin and eosin–stained slide was used to define regions of tumor and to confirm the absence of tumor cells in normal tissue sections. Genomic DNA was isolated from macrodissected tissue containing at least 80% of neoplastic cells (tumor), or no visible tumor cells (normal) using the QIAamp DNA FFPE tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. A final four MTC DNA samples were derived from a previous study (23).

RET^{M918T} *gene mutation analysis in sporadic MTC tumor samples*

Mutation detection was performed through the Characterized Cell Line Core facility using a CLIA-certified Sequenom MALDI TOF MassArray system (Sequenom, San Diego, CA). Assay primers used were designed to specifically detect the presence or absence of *RET_M918T_T2753C*. The somatic assignment of *RET*^{M918T} mutation was based on the lack of mutation in paired peripheral blood cells or normal thyroid DNA performed in all patients.

Detection of CDKN2C *(p18) copy number loss*

To facilitate high-throughput analysis of *CDKN2C* loss at reduced expense, the authors collaborated with the Characterized Cell Line Core facility to develop a multiplexed SNP genotyping assay using the Sequenom MALDI TOF MassArray system. The assay included 22 SNPs mapping a 223,049 bp region from 51,086,606 to 51,309,654 on chromosome 1p (NCBI36/hg18; see Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/thy). The *CDKN2C* gene is centrally located within this region (chr1:51,206,196–51,212,897). Samples with paired normal thyroid tissue were tested using this Sequenom platform. Within this group, 7/47 samples were noninformative for *CDKN2C* single copy loss. Follow-up testing on these seven samples along with 15 additional samples lacking paired normal tissue was performed using the qBiomarker Copy Number PCR Assay (QIAGEN) with four *CDKN2C* targeted primer sets (VPH101-0257177A, VPH101-0257178A, VPH101-0257200A, and VPH101-0257201A) according to the manufacturer's instructions.

CDKN2C *gene mutation analysis in sporadic MTC tumor samples*

In those patients in which *CDKN2C* loss was detected, tumor DNA sequencing was performed to distinguish haploinsufficiency from *CDKN2C* inactivation occurring through mutation of the remaining gene copy. Targeted mutational analysis was performed on polymerase chain reaction (PCR) products derived from *CDKN2C* exons 1 and 2. The coding regions were amplified using primers specific to exon 1 (forward: GTA TTT ACT ACC AAG CTC TAC TCC AG; reverse: GTA CGT CAT TTT GAG AAG TTG CAT CC) and exon 2 (forward: GTC TTC TAC TTT CAC CTA CTA GCA C; reverse: GCT GCT TAA CAT ATG ACA GAA CTG). The amplified PCR products were treated with shrimp alkaline phosphatase and exonuclease 1 (37°C for 1 h, 72°C for 15 min) before submission to the Sequencing and Microarray Core facility. It was not possible to perform sequence analysis for a single sample because of exhaustion of the DNA sample for other analyses.

Statistics

Fisher's exact test was used to evaluate the association between two categorical variables. Wilcoxon's rank sum test was used to assess the difference in a continuous variable between patient groups. OS was estimated using Kaplan–Meier survival method, and the difference in OS between patient groups was assessed by the log-rank test. Univariate Cox proportional hazards model was fitted to evaluate the effect of a prognostic factor on OS. All statistical analyses were performed using SAS v9.3 for Windows (SAS Institute, Inc., Cary, NC). A p-value of 0.05 was used to determine significance.

Results

Clinical evaluation

Sixty-two MTC cases with an initial surgery date ranging from 1983 to 2009 met the inclusion criteria. Dividing this time period into thirds, five patients were diagnosed from 1983 to 1991, 24 from 1992 to 2000, and 33 from 2001 to 2009. Detailed demographic and pathology characteristics as well as clinical course details are provided in Table 1. The median age at initial surgery of the 62 MTC patients was 53 years (range 22–81 years). Thirty-six (58%) patients were

Table 1. Demographic and Pathology Characteristics and Clinical Course of All Cohort Patients with Medullary Thyroid Cancer and Divided by Tumor Somatic 918 Mutation Status

Variable	*Total (%),* n = *62*	*RET*^{M918T} *(negative),* n = *31*	*RET*^{M918T} *(positive),* n = *31*	p-*Value*
Sex				1.00
Female	26 (42%)	13 (42%)	13 (42%)	
Male	36 (58%)	18 (58%)	18 (58%)	
Age at first surgery (years), median (range)	53 (22–81)	59 (22–81)	51 (25–77)	0.04
Preoperative calcitonin (pg/mL), median (range)	5150 (6–129,780)	1154 (6–92,230)	8660 (682–129,780)	0.04
Tumor size (mm), median (range)	30 (6–145)	30 (11–145)	32 (6–70)	0.72
Extrathyroidal extension				0.35
Yes	29 (57%)	12 (50%)	17 (63%)	
No	22 (43%)[a]	12 (50%)	10 (37%)	
Invasion of adjacent structures				0.81
Yes	13 (23%)	6 (21%)	7 (24%)	
No	44 (77%)[b]	22 (79%)	22 (76%)	
T status				0.57
1	13 (22%)	7 (24%)	6 (20%)	
2	17 (28%)	10 (33%)	7 (23%)	
3	20 (33%)	10 (33%)	10 (34%)	
4	10 (17%)[c]	3 (10%)	7 (23%)	
N status				0.04
0	13 (22%)	10 (35%)	3 (10%)	
N1a	5 (8%)	3 (10%)	2 (6%)	
N1b	42 (70%)[c]	16 (55%)	26 (84%)	
M status				0.11
0	49 (80%)	27 (90%)	22 (71%)	
1	12 (20%)[d]	3 (10%)	9 (29%)	
Stage				0.06
1	4 (7%)	3 (11%)	1 (3%)	
2	9 (16%)	7 (26%)	2 (7%)	
3	1 (2%)	1 (4%)	0 (0%)	
4a	31 (54%)	13 (48%)	18 (60%)	
4c	12 (21%)[b]	3 (11%)	9 (30%)	
Development of local regional recurrence				0.49
Yes	16 (30%)	7 (26%)	9 (35%)	
No	37 (70%)[e]	20 (74%)	17 (65%)	
Development of metachronous distant disease*				0.28
Yes	20 (32%)	8 (26%)	12 (39%)	
No	42 (68%)	23 (74%)	19 (61%)	
Vital status at last follow-up				**
Alive, disease free	17 (27%)	11 (38%)	6 (18%)	
Alive, evidence of disease	18 (29%)	6 (21%)	12 (36%)	
Dead	27 (44%)	12 (41%)	15 (45%)	

[a]Eleven patients with missing data.
[b]Five patients with missing data.
[c]Two patients with missing data.
[d]One patient with missing data.
[e]Nine patients with missing data.
*Independent of initial M status.
**The data were analyzed as time-to-event endpoints for death and disease death.

male. The median tumor size was 30 mm (range 6–145 mm) with 29 (57%) possessing extrathyroidal extension. Nodal and distant metastasis at presentation was found in 47/60 (78%) and 12/61 (20%) patients, respectively. Of the 57 patients with sufficient available pathology data, according to TNM classification, four patients had Stage 1 (T1N0M0) disease, nine had Stage 2 (T2-T3N0M0), one had Stage 3 (T1-T3N1aM0), 31 had Stage 4a (T1-T4aN1bM0), and 12 had Stage 4c (any M1) disease. Median follow-up time was 9.8 years (range 1.1–27.8 years) for the censored observations. At the conclusion of follow-up, locoregional disease recurrence had occurred in 16 (30%), and development of metachronous distant disease in 20 (32%), either additional sites of distant metastasis if M1 at original

Table 2. Demographic and Pathology Characteristics of Patients with Medullary Thyroid Cancer by Tumor Somatic CDKN2C Copy Number Status

Variable	*CDKN2C 2N,* n = *50*	*CDKN2C loss,* n = *12*	p-*Value*
Sex			0.53
Female	20 (40%)	6 (50%)	
Male	30 (60%)	6 (50%)	
Age at surgery (years), median (range)	52 (22–81)	61 (40–77)	0.18
Preoperative calcitonin, median (range)	3850 (6–129,780)	8980 (656–92,230)	0.24
Tumor size (mm), median (range)	30 (6–145)	40 (10–70)	0.17
Extrathyroidal extension			1.00
Yes	23 (58%)	6 (55%)	
No	17 (42%)[a]	5 (45%)	
Invasion of adjacent structures			0.10
Yes	8 (17%)	5(45%)	
No	38 (83%)[b]	6 (55%)	
T status			0.87
1	11 (23%)	2 (17%)	
2	14 (29%)	3 (25%)	
3	16 (33%)	4 (33%)	
4	7 (15%)[c]	3 (25%)	
T status (combined)			0.75
1/2	25 (52%)	5 (42%)	
3/4	23 (48%)	7 (58%)	
N status			0.13
0	8 (17%)	5 (42%)	
1a	4 (8%)	1 (8%)	
1b	36 (75%)[c]	6 (50%)	
M status			0.003
0	43 (88%)	6 (50%)	
1	6 (12%)[d]	6 (50%)	
Stage			0.0009
1	2 (4%)	2 (17%)	
2	6 (13%)	3 (25%)	
3	1 (2%)	0	
4a	30 (67%)	1 (8%)	
4c	6 (13%)[b]	6 (50%)	
RET^{m918T} status			1.00
Positive	25 (50%)	6 (50%)	
Negative	25 (50%)	6 (50%)	
Development of local regional recurrence			0.31
Yes	14 (34%)	2 (17%)	
No	27 (66%)[e]	10 (83%)	
Development of metachronous distant disease*			0.31
Yes	18 (36%)	2 (17%)	
No	32 (64%)	10 (83%)	
Vital status at last follow-up			**
Alive, disease free	14 (18%)	3 (25%)	
Alive, evidence of disease	18 (36%)	0	
Dead	18 (36%)	9 (75%)	

[a]Eleven patients with missing data.
[b]Five patients with missing data.
[c]Two patients with missing data.
[d]One patient with missing data.
[e]Nine patients with missing data.
*Independent of initial M status.
**The data were analyzed as time-to-event endpoints for death and disease death.

staging or new distant disease if originally M0. Twenty (32%) patients underwent treatment with systemic therapy: seven patients with standard chemotherapy, nine with targeted molecular therapy, and four with both at staggered intervals.

At the conclusion of the follow-up interval, 17 patients were free of disease, 18 had evidence of disease, and 27 had died. Sixteen deaths were from MTC, six from other causes, and five from unknown causes. Six of the deaths were known to be non-MTC causes, one secondary to metastatic breast

cancer, and five related to other comorbidities in patients who had no evidence of MTC on last follow-up. Of the five patients with unknown causes for death, two died at ages 86 and 83 with small-volume pulmonary disease and cervical disease, respectively. One patient died at 83 years of age with no evidence of MTC. The remaining two patients included one patient who died at 58 years of age and had last been seen at follow-up 24 months earlier with progressing lung metastasis, and one who died at 76 years of age with respiratory complications, three months after a complicated cervical and mediastinal surgery requiring tracheostomy with known residual intrathoracic disease.

RET^{M918T} *mutation and* CDKN2C *copy number loss*

The association of somatic RET^{M918T} mutation status with pathology, clinical features, and outcome is described in Table 1. Within the cohort, 31/62 (50%) patients had a somatic RET^{M918T} mutation. The presence of a somatic RET^{M918T} mutation was significantly associated with younger age at first surgery (median 51 vs. 59 years; $p = 0.04$), higher preoperative calcitonin levels (8660 vs. 1154 pg/mL; $p = 0.04$), and presence of nodal metastasis (90% vs. 65%; $p = 0.04$). Sex, extrathyroidal extension, T and M status, and overall AJCC staging were not statistically different among patients with and without somatic RET^{M918T} mutations. There was no association between era of diagnosis and RET^{M918T} status (data not shown). No difference was noted between development of locoregional disease and development of initial or additional sites of distant metastasis based on the presence of a RET^{M918T} mutation.

The status of a *CDKN2C* copy number loss was examined with respect to pathology, clinical features, and outcome as shown in Table 2. Twelve (19%) patients had allelic loss encompassing the *CDKN2C* gene, of whom six also had a RET^{M918T} mutation. It was possible to confirm the absence of a second mutation event in 11/12 tumor samples as only normal *CDKN2C* gene sequence was observed (see Methods); the 12th sample was unable to be tested due to lack of specimen. Sex, age at first surgery, preoperative calcitonin, and T and N stage were not different among patients with and without *CDKN2C* loss. However, unlike RET^{M918T} status, the presence of *CDKN2C* loss was associated with worse M stage (50% vs. 12%; $p = 0.003$) and overall AJCC stage ($p = 0.0009$). No difference was noted between development of locoregional disease and development of initial or additional sites of distant metastasis based on the presence of a *CDKN2C* loss.

Predictors of outcome in sporadic MTC patients

In order to determine the role of RET^{M918T} mutation and *CDKN2C* loss in MTC tumorigenesis, predictors of overall survival in the patient cohort were examined (Table 3). Univariate analysis included the following variables: sex, age, preoperative calcitonin level, tumor size, presence of extrathyroidal extension, invasion of adjacent structures, TNM stage, RET^{M918T} mutation, and *CDKN2C* copy number status (both alone and combined). Multivariate analysis was not performed, given the limited number of events. Invasion of tumor into of adjacent structures (hazard ratio [HR] 4.56 [confidence interval (CI) 1.89–11.04]; $p = 0.0008$), worse T status (HR 3.46 [CI 1.00–11.91]; $p = 0.04$), worse M status (HR 9.31 [CI 3.89–22.27]; $p < 0.0001$), and worse overall stage (HR 8.26 [CI 1.05–64.74]; $p = 0.0002$) were associated with shorter overall survival, as was a higher preoperative calcitonin level (HR 2.97 [CI 1.11–7.94]; $p = 0.30$). The presence of *CDKN2C* loss (HR 4.74 [CI 2.01–11.19]; $p = 0.0004$), and a combination of RET^{M918T} mutation and *CDKN2C* loss (HR 11.24 [CI 3.28–38.53]; $p = 0.0006$) were observed to be highly significant predictors of outcome. Notably, the RET^{M918T} mutation alone was not associated with worse survival (HR 1.33 [CI 0.62–2.86]; $p = 0.460$).

The median overall survival for the entire cohort was 15.9 years [CI 11.9–NA]. Median overall survival of patients with a somatic RET^{M918T} mutation versus those without a somatic RET^{M918T} mutation was 17.24 [CI 9.31–NA] versus 15.87 [CI 11.92–NA] years ($p = 0.46$). Median overall survival of patients with a somatic *CDKN2C* copy number loss versus those without a somatic *CDKN2C* loss was 4.14 [CI 1.93–NA] versus 18.27 [CI 13.46–NA] years ($p < 0.0001$; Fig. 2). Median overall survival of patients with a combined somatic

Table 3. Univariate Analysis of Overall Survival of Patients with Sporadic Medullary Thyroid Carcinoma

Variable	*HR*	*CI*	p-*Value*
Sex			0.609
Female vs. male	0.61	0.27–1.37	
Age			0.152
≥53 vs. <53 years	1.79	0.81–3.95	
Preop calcitonin			0.030
≥5150 vs. <5150 pg/mL	2.97	1.11–7.94	
Tumor size			0.221
≥30 mm vs. <30 mm	1.76	0.71–4.38	
Extrathyroidal extension			0.022
Yes vs. no	3.62	1.21–10.85	
Invasion of adjacent structures			0.0008
Yes vs. no	4.56	1.89–11.04	
T status			0.040
T4 vs. T1	3.46	1.00–11.91	
T3 vs. T1	1.29	0.40–4.17	
T2 vs. T1	0.70	0.17–2.84	
N status			0.800
N1b vs. N0	1.44	0.49–4.27	
N1a vs. N0	1.31	0.28–6.04	
M status			<0.0001
M1 vs. M0	9.31	3.89–22.27	
Stage			0.0002
4c vs. 1	8.26	1.05–64.74	
4a vs. 1	0.98	0.12–7.87	
3 vs. 1	—		
2 vs.1	1.16	0.12–11.38	
RET^{M918T} status			0.460
+ vs. −	1.33	0.62–2.86	
CDKN2C status			0.0004
Loss vs. no loss	4.74	2.01–11.19	
RET^{M918T}/*CDKN2C* status			0.0006
+/+ vs. −/−	11.24	3.28–38.53	
−/+ vs. −/−	3.25	0.95–11.14	
+/− vs. −/−	1.21	0.47–3.07	

HR, hazard ratio; CI, confidence interval.

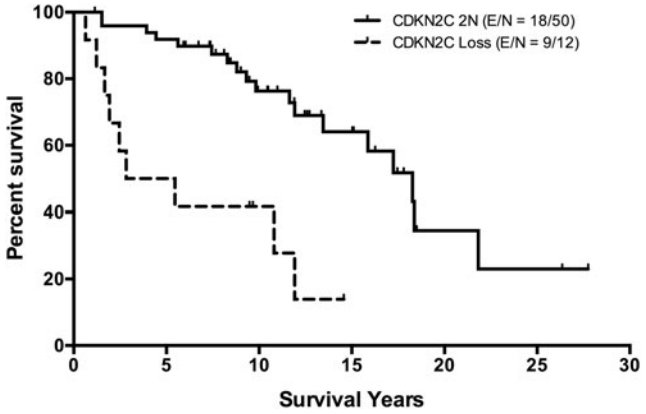

FIG. 2. Overall survival (OS) of medullary thyroid carcinoma (MTC) patients with somatic *CDKN2C* copy number loss versus those patients with somatic tumor *CDKN2C* 2N. Median follow-up 10.5 years; median OS *CDKN2C* copy number loss versus *CDKN2C* 2N 4.14 [confidence interval (CI) 1.93–NA] versus 18.27 [CI 13.46–NA] years ($p < 0.0001$).

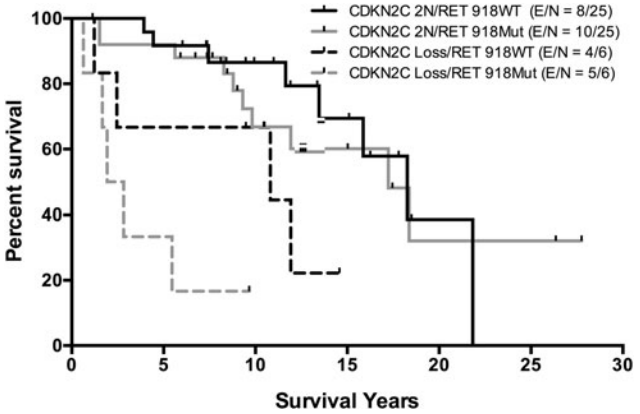

FIG. 3. OS of MTC patients with a combined somatic *CDKN2C* copy number loss and somatic RET^{M918T} mutation versus somatic *CDKN2C* copy number loss and wild type (WT) somatic RET^{M918T} versus somatic *CDKN2C* 2N and somatic RET^{M918T} mutation versus somatic *CDKN2C* 2N and WT somatic RET^{M918T}. Median follow-up 10.5 years; median OS of patients with a combined somatic *CDKN2C* copy number loss and RET^{M918T} mutation versus *CDKN2C* copy number loss and WT somatic RET^{M918T} versus *CDKN2C* 2N and RET^{M918T} mutation versus *CDKN2C* 2N and WT somatic RET^{M918T} was 2.38 [CI 1.67–NA] years versus 10.81 [CI 2.46–NA] versus 17.24 [CI 9.82–NA] versus 18.27 [CI 13.46–NA] years ($p < 0.0001$).

RET^{M918T} mutation and *CDKN2C* copy number loss versus no somatic RET^{M918T} mutation and *CDKN2C* loss versus somatic RET^{M918T} mutation and no *CDKN2C* loss versus no somatic RET^{M918T} mutation and no *CDKN2C* loss was 2.38 [CI 1.67–NA] years versus 10.81 [CI 2.46–NA] versus 17.24 [CI 9.82–NA] versus 18.27 [CI 13.46–NA] years ($p < 0.0001$; Fig. 3). DSS was evaluated in a similar manner with the same significant variable relationships as described for overall survival.

Discussion

This study evaluated the relationship of *CDKN2C* haploinsufficiency in sporadic MTC tumors with clinical aggressiveness of disease. Haploinsufficiency was defined by loss of a single copy without concomitant mutation. Twelve of 62 (19%) sporadic MTC tumors had a *CDKN2C* loss, an incidence similar to the reported loss of chromosome 1p32 regions that contains *CDKN2C* (summarized in Table 4) (22,23,29–34). The presence of *CDKN2C* loss showed a significant association with the incidence of distant metastasis at presentation, as well as with decreased DSS and OS. While a relationship between *CDKN2C* loss and various adverse clinical factors has been suggested in previous small case reports (33, 34), this study represents the first evaluation of its relationship with survival. Additionally, half the patients in the present cohort had a RET^{M918T} mutation detected in their tumor; the combination of a somatic RET^{M918T} alteration with the presence of *CDKN2C* loss further decreased a patient's disease specific and overall survival, as shown in Figure 2. While these relationships are compelling, the relatively small number of cases reported here caution against making definitive conclusions of the pathogenicity of CDKN2C loss and its association with a RET^{M918T} mutation. However, given the rarity of MTC, larger single-institution studies are not readily feasible; these initial findings require validation in further cohorts.

Why study copy number in MTC and specially target *CDKN2C* haploinsufficiency? The potential role of somatic copy number alterations (SCNAs) has been an underinvestigated mechanism of tumorigenesis in general (24). Furthermore, only *RET*, *HRAS*, and *KRAS* have been identified as clear drivers of tumorigenesis, accounting for approximately 60% of MTC. Therefore, SCNA and epigenetic regulation should be considered as an additional potential source of oncogenicity (24,35). A fundamental tenet of this proposal is that in the absence of well-defined heterozygous markers, single copy gene loss would go undetected by the most common current mutation investigational mechanisms. Thus, in the common paradigm of simply searching for second hit mutations without considering the cellular impact of SCNAs, causative roles for haploinsufficiency in tumorigenesis are likely underestimated (24). The role of SCNA change in human MTC has been evaluated in several small independent studies, with losses in 1p, 19p, and 22 representing the most consistent SCNAs associated with this malignancy (Table 4). Of the several hundred candidate genes that exist in these regions, particular focus has been paid to the candidate tumor suppressor gene *CDKN2C* located on 1p32 (31,36). A specific haploinsufficient loss-of-function role for *CDKN2C* in MTC is supported by the mouse knockout model (*RET2B;p18*$^{+/-}$), which expresses human RET^{M918T} from a transgene in a genetic background lacking one copy of *Cdkn2c* (p18) (14). Complete loss of *CDKN2C* is associated with hyperplastic growth within murine endocrine organs, including the C-cells, along with a low incidence of MTC, which is greatly enhanced by coincident *CDKN1B* (p27) haploinsufficiency or complete loss (37). As CDKNs function as regulators of RB1 (Fig. 1), it is not surprising that Rb1 knockout mice also develop MTC (38–41).

This study determined the frequency of *CDKN2C* loss (using targeted analysis) in a cohort of patients diagnosed with sporadic MTC and examined the relationship of gene

TABLE 4. DETAILED COMPARISON OF CDKN2C LOSS WITH PREVIOUSLY PUBLISHED 1P32 LOSS STUDIES

Study	*Yang*[28] et al., *1990*	*Moley*[29] et al., *1992*	*Mulligan*[30] et al., *1993*	*Hemmer*[31] et al., *1999*	*Frisk*[32] et al., *2001*	*Marsh*[33] et al., *2003*	*Ye* et al.,[20] *2008**	*Flicker*[19] et al., *2012*	*Grubbs* et al., *2016*	*Total*
No. of cases										
Total	7	24	28	10	23	37	30	52	62	273
Hereditary (%)	4 (57%)	19 (79%)	18 (64%)	NA	3 (13%)	8 (22%)	10 (33%)	11 (21%)	0	73 (28%)
Sporadic (%)	3 (43%)	5 (21%)	10 (36%)	NA	20 (87%)	29 (78%)	20 (67%)	41 (79%)	62 (100%)	190 (72%)
1p32 region loss										
Total (%)	4/7 (57%)	3/24 (13%)	7/28 (25%)	1/10 (10%)	0/23 (0%)	5/37 (14%)	5/30 (23%)	18/52 (35%)	12/62 (19%)	55/273 (20%)
Hereditary (%)	2/4 (50%)	3/19 (16%)	6/18 (33%)	NA	0/3 (0%)	1/8 (13%)	0/10 (0%)	2/11 (18%)	NA	14/73 (19%)
Sporadic (%)	2/3 (67%)	0/5 (0%)	1/10 (10%)	NA	0/20 (0%)	4/29 (14%)	5/20 (25%)	16/41 (39%)	12/62 (19%)	40/190 (21%)
RET Mut+	NA	NA	NA	NA	0/10 (0%)	2/13 (15%)	2/10 (20%)	6/15 (40%)	6/12 (50%)	16/60 (27%)
RET Mut–	NA	NA	NA	NA	0/10 (0%)	2/16 (13%)	3/10 (30%)	10/26 (38%)	6/40 (15%)	21/102 (21%)
Outcome studies	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Not specifically evaluated	Not evaluated	No correlation with outcome measures studied: tumor size, # +LN	Correlation with T stage, M stage, DSS, and OS	

*Data for 1p32 loss was not specifically reported
NA, not available; DSS, disease-specific survival; OS, overall survival.

loss with tumor presentation, aggression, and patient survival. It also evaluated for a potential association of RET^{M918T} mutation with *CDKN2C* loss and tumor behavior, which is important given that these two alterations have usually appeared concomitantly in previous mouse and human studies when MTC develops (14,15). The presence of *CDKN2C* loss had a significant relationship with M1 status in univariate analysis, which directly influenced its association with the TNM stage of 4c (Table 2). M stage and *CDKN2C* SCNA status were also independently associated with overall survival (Table 3). The small cohort with a limited number of events (deaths) prevented use of adjustment models in the analysis, an acknowledged limitation of this study. Other study variables associated with decreased overall survival, such as T status and gross extrathyroidal extension invading into adjacent structures, have similarly been found to be significant in other published reports (42–44). These variables, which may be viewed as measures of aggressiveness, were not associated with *CDKN2C* SCNA status. The observation that *CDKN2C* loss was not associated with locoregional variables of aggressiveness but was with distant metastasis may point to the mechanism MTC tumor spread. Importantly, the gene that serves as the primary target of CDKN action, *RB1*, has well established roles in promoting tumor angiogenesis and distant metastasis (45).

In this study, the addition of a RET^{M918T} mutation in the presence of a *CDKN2C* loss appeared to worsen the prognosis. Inactivating *CDKN2C* mutations have been found to coexist with activating *RET* mutations in both human MTCs and pheochromocytomas (15), supporting the concept that *CDKN2C* functions as a haploinsufficient tumor suppressor gene in the presence of other defects upregulating phosphoRB-mediated cell cycle progression. In the $RET2B;p18^{+/-}$ mouse model described above (14), independently, either overexpression of RET^{M918T} or loss of a single copy of *CDKN2C* lead only to C-cell hyperplasia in mice. However, their combination is associated with a dramatic increase the risk of MTC development as well as enhanced tumor progression.

The tumor mutational profile of this cohort is directly influenced by the patients who are referred to the authors' tertiary care center; the proportion of patients presenting with nodal disease is 87% compared with 59% of the 1957 patients with known nodal status in the National Cancer Database (NCDB) (42). Additionally, 20% of the population presents with distant metastatic disease compared with 11% in the NCDB. While these data suggest that the present cohort presents with more aggressive disease than the national population, univariate analysis findings are similar to the NCDB unadjusted overall survival in that M status has by far the most significant relationship with survival of evaluated variables. Thus, while the present cohort has more aggressive disease, it behaves similarly to other reported patient groups with aggressive disease. The number of RET^{M918T} mutations and *CDKN2C* loss found in the present population are similar in proportion to others reported in the literature—small groups that may be equally influenced by referral biases. This observation does not lessen the importance of these findings, but rather it cautions us when trying to extrapolate mutational percentages to the larger population.

In conclusion, this study provides clinical context for a molecular alteration, *CDKN2C* loss, which has been described by others to be present in MTC. It was observed that

genetic loss of a region containing *CDKN2C* has a relationship with distant metastasis and OS in the cohort examined that is enhanced by a concomitant RET^{M918T} mutation. Given the small number of patients included in the study, these findings should be considered as exploratory in nature but nonetheless important to pursue because defining pathways of disease progression allow the development of targeted cancer therapies. Indeed, a recent comprehensive genomic profiling of 34 aggressive MTC cases further supports this treatment paradigm (46). This report found alterations in the Rb1 pathway genes, including *CDK4*, *CDK6*, *CDKN2A/B*, or *CDKN2C*, in 21% of specimens, often with coincident *RET*-activating mutations, suggesting hyperactivation of the cyclin D-dependent kinases CDK4 and CDK6. Targeting cancers through CDK inhibition to regain control of the cell cycle is currently being evaluated in several clinical studies (47–50). The findings support the study of approved RET targeting agents in combination with CDK inhibitors in preclinical studies of MTC. This approach may ultimately best benefit a subpopulation of MTC patients with RET^{M918T} mutations and concomitant *CDKN2C* loss.

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Author Disclosure Statement

No competing financial interests exist for any of the authors.

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