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Clinical Research Article

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CD133 Expression in Medullary Thyroid Cancer Cells Identifies Patients with Poor Prognosis

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Abbreviations: AJCC, American Joint Committee on Cancer; CNIO, Spanish National Cancer Research Centre; IHC, immunohistochemistry; MEN2, multiple endocrine neoplasia type 2; MTC, medullary thyroid cancer; PCR, polymerase chain reaction; RTK, receptor tyrosine kinase.

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Abstract

Context: The identification of markers able to determine medullary thyroid cancer (MTC) patients at high-risk of disease progression is critical to improve their clinical management and outcome. Previous studies have suggested that expression of the stem cell marker CD133 is associated with MTC aggressiveness.

Objective: To evaluate CD133 impact on disease progression in MTC and explore the regulatory mechanisms leading to the upregulation of this protein in aggressive tumors.

Patients: We compiled a series of 74 MTCs with associated clinical data and characterized them for mutations in *RET* and *RAS* proto-oncogenes, presumed to be related with disease clinical behavior.

Results: We found that CD133 immunohistochemical expression was associated with adverse clinicopathological features and predicted a reduction in time to disease progression even when only RET-mutated cases were considered in the analysis (log-rank test $P < 0.003$). Univariate analysis for progression-free survival revealed CD133 expression and presence of tumor emboli in peritumoral blood vessels as the most significant prognostic covariates among others such as age, gender, and prognostic stage. Multivariate analysis identified both variables as independent factors of poor prognosis (hazard ratio = 16.6 and 2; $P = 0.001$ and 0.010, respectively). Finally, we defined hsa-miR-30a-5p, a miRNA downregulated in aggressive MTCs, as a CD133 expression regulator. Ectopic expression of hsa-miR-30a-5p in MZ-CRC-1 (RET^{M918T}) cells significantly reduced CD133 mRNA expression.

Conclusions: Our results suggest that CD133 expression may be a useful tool to identify MTC patients with poor prognosis, who may benefit from a more extensive primary surgical management and follow-up.

Key Words: RET, RAS, immunohistochemistry, disease progression, calcitonin expression and tumor emboli

Medullary thyroid cancer (MTC) is a rare disease that accounts for 5% to 10% of all thyroid carcinomas. Despite its low prevalence, MTC is associated with a high mortality rate when diagnosed in advanced stages (10-year survival rate of 21%) (1). This neuroendocrine malignancy is caused by the abnormal proliferation of C-cells, which represent 1% of thyroid gland cells. Approximately 25% of MTCs are hereditary as part of the multiple endocrine neoplasia type 2 (MEN2) syndrome, and are classified according to their clinical manifestations into MEN2A and MEN2B (1).

Activating mutations in the REarranged during Transfection gene (*RET*) are present in 98% of MEN2 forms (2). The *RET* (10q11.2) proto-oncogene encodes a receptor tyrosine kinase (RTK) that activates multiple intracellular signals related to C-cell differentiation and homeostasis in a ligand-dependent manner (1-3). Although all MTC-associated *RET* mutations constitutively activate the RTK, the intensity and outcome of aberrant signaling are mutation-specific (4-6), which probably explains the strong genotype-phenotype association observed for this disease. Indeed, current clinical management of MEN2 patients is based on *RET* genotype and the associated risk category (highest/high/moderate) for developing an aggressive MTC (1, 7). However, recent reports claim that the current risk stratification system predicts MTC age of onset (early/late) but not the clinical course of the disease, and recommend a modification of risk classification terminology for hereditary MTCs (8, 9).

RET^{M918T} is the mutation with the highest associated risk to develop an aggressive MTC in MEN2 patients and it is also the most prevalent mutation in sporadic MTCs (33.3%-39.2%) (10-13), followed by mutations in *K-* and *H-RAS* proto-oncogenes (10.9%-26.2%) (14-17) and other *RET* mutations (9%-19.6%) (11, 13), which are mutually exclusive. As in hereditary disease, the RET^{M918T} mutation has been correlated with poorer prognosis in sporadic MTCs as compared with other genetic classes (13, 18); still, RET^{M918T}–negative tumors can also be highly aggressive malignancies, pointing to molecular factors other than *RET* mutation status and genotype as determinants of MTC tumor virulence.

High-throughput technologies have led to the characterization of the genomic features of multiple cancer types and play a key role in the identification of diagnostic, prognostic, and therapeutic biomarkers that have improved clinical management of cancer patients (19). However, the low prevalence of MTCs has challenged the use of these technologies in MTC studies and few reports have identified molecular events with a potential clinical application (20-22). Several years ago, our group characterized the transcriptional profile of 49 MTCs and identified a genetic context–dependent differential expression of the *PROM1* gene, which was overexpressed in RET^{M918T} tumors and related to cell survival in human MTC cell lines (23). A more recent study in a cohort of 49 Chinese MTC patients found that the expression of PROM1 was an unfavorable

predictor for overall survival (24), highlighting the potential clinical value of this marker. *PROM1* encodes a pentaspan transmembrane glycoprotein localized in membrane protrusions, known as CD133. It has been described that CD133 maintains stem cell properties of cancer cells by suppressing differentiation pathways (25, 26), and its expression has been related to chemoresistance in MTC cell lines (27). The deregulation of certain miRNAs has been associated with CD133 overexpression in different tumor types (28-30); however, the regulatory mechanisms that induce CD133 expression in MTC cells are poorly understood. In this study, we aimed to determine the significance of PROM1/CD133 expression as a predictor of prognosis in a previously unpublished large series of MTCs.

Materials and Methods

Human MTC tissue samples

Seventy-four formalin-fixed paraffin-embedded (FFPE) medullary thyroid carcinoma tumors from unrelated patients were included in the study. The MTC series was independent of the one used by Maliszewska et al (23) and Mancikova et al (20). Samples were collected through a Spanish National Hospital network including Hospital Universitario La Paz, Hospital General Universitario Gregorio Marañon, Hospital Universitario de La Princesa, Hospital Universitario San Cecilio, Hospital Universitario 12 de Octubre, Hospital General Universitario de Ciudad Real, Hospital Clínico San Carlos, Hospital Universitario Príncipe de Asturias, Complejo Hospitalario Universitario de Albacete, Hospital Universitario Infanta Sofía, Hospital Virgen de la Salud, and Hospital Universitario Puerta de Hierro, with the collaboration of the Spanish National Cancer Research Centre (CNIO) Tumor Bank. Relevant clinical data are summarized in Supplementary Table 1 (31). The study was approved by the respective institutional review boards. Written informed consent was obtained from all patients included in the study.

RET and *RAS* mutation analysis

Genomic DNA from all available peripheral blood samples was isolated using the MagNA Pure 24 System (Roche) or the Maxwell RSC Whole Blood DNA kit and System (Promega). Genomic DNA from FFPE specimens was isolated with the DNeasy kit (Qiagen) or the TruXTRAC FFPE DNA Kit (Covaris), respectively, following the manufacturers' protocols. The genetic screening of *RET* and (*H*-, *K*-) *RAS* mutations was prioritized on the tumors and performed by Sanger sequencing of MTC-related exons (exons 8, 10, 11, and 13 to 16 for *RET*; exons 2 and 3 for *H*- and *K-RAS*). The identified mutations were tested by polymerase chain reaction (PCR) of the corresponding exon in the germline DNA to determine whether the mutations were somatic or germline and, therefore, whether the MTC was sporadic or hereditary. As expected, all identified *RAS* mutations were somatic. Sequences of the primers and PCR annealing temperatures are summarized in Supplementary Methods Table 1 (31). Tumors without mutations in any of the tested exons (nonRET/nonRAS genotype) were re-analyzed with a modified version of the targeted next-generation sequencing custom panel PheoSeq (32), which includes exons 5, 8-11, and 13-16 of *RET* (ENST00000355710), and exons 2 and 3 of *HRAS* (ENST00000451590) and *KRAS* (ENST00000311936), to overcome Sanger sequencing limited sensitivity for somatic mutation detection.

Immunohistochemical assay and evaluation

Consecutive 3-µm thick complete sections of the paraffin-embedded tissues were mounted on Thermo Scientific™ SuperFrost™ plus slides and dried overnight. Slides were deparaffinized in xylene and rehydrated through a series of graded ethanol solutions and finally rinsed in water. Immunohistochemistry reactions were performed in an automated immunostaining platform (Autostainer Link 48, Dako) following standard instructions. Antigen retrieval was first performed using the appropriate buffer (high pH buffer, Dako) and endogenous peroxidase was blocked with 3% hydrogen peroxide solution in methanol for 15 minutes. Then, slides were incubated with rabbit monoclonal anti-human PROM1/CD133 (D4W4N, 1/700, Cell Signaling, #86781) or FLEX rabbit polyclonal anti-human calcitonin (Dako, ready to use, IR515). After the primary antibody, slides were incubated with the visualization systems (EnVision FLEX + Rabbit linker, Dako) conjugated with horseradish peroxidase.

Slides were developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (DAB+, Dako) and nuclei were counterstained with Carazzi's hematoxylin. The slides were dehydrated, cleared and mounted with a permanent mounting medium for microscopic evaluation. Positive control sections known to be primary antibody positive were included for each staining run. Paraffin-embedded human kidney was used as control. As previously described (33), CD133 antibody stained parietal layer of Bowman's capsule and renal tubules (Supplementary Methods Fig. 1a) (31).The entire immunochemistry protocol was carried out by the CNIO Histopathology Core Unit.

CD133 immunostaining was evaluated by an expert pathologist (E.C.), who was blinded to the clinical and molecular data. Expression was considered positive when > 5% of the tumor cells showed moderate (++) or high

(+++) staining intensity by examining at least 10 fields of complete sections by high-power (40x) microscopy (Leica DMLB). Consecutive sections of a subset of tumors (n = 15, 9 CD133-positive and 6 CD133-negative tumors) were stained for calcitonin and evaluated by E.C. In addition, a Q-PCR for CD133 encoding gene *PROM1* expression was performed in 5 CD133-positive and 4 CD133-negative MTCs to validate CD133 immunohistochemical results (Supplementary Methods Fig. 1b) (31).

Tumor RNA extraction and calcitonin gene expression quantification

Total RNA from at least 2 tissue cores of selected tumor areas was isolated from a subset of MTCs (n = 16) using the Maxwell RSC RNeasy kit and instrument (Promega) following the manufacturer's protocols. A calcitonin gene expression probe was included in a TruSeq Targeted custom panel (Illumina) together with 227 probes for 187 genes. RNA sequencing data were DESeq2-normalized (34). Values for calcitonin gene expression were $\log_2$ converted.

In silico identification of hsa-miR-30a as a mechanism of regulation of PROM1/CD133 expression

Combined analysis of the methylome, miRnome and transcriptome of human MTCs recently identified a genetic context–dependent activation of several regulatory nodes (20). Hsa-miR-30a-5p was the core of one of these regulatory nodes, which showed up as one of the least expressed miRNAs in RET^{M918T} MTCs compared to the other genotypes. The Targetscan database (TargetScan human 7.2) (35), which predicts regulatory targets of mammalian microRNAs, shows that the hsa-miR-30a-5p-site is one of the 3 conserved miRNAs-sites in the *PROM1* 3' UTR sequence and has the highest P_{ct} score (P_{CT} = 0.78), which measures the probability of a miRNA site to be functional.

Human MTC cell lines and culture conditions

MTC cell line TT (RET^{C634Y}) was obtained from ECACC and MZ-CRC-1 (RET^{M918T}) was kindly provided by Dr. James Fagin (Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York) and validated by *RET* exon 11 and 16 sequencing. Both cell lines were found to be negative for mycoplasma by the CNIO Monoclonal Antibody Unit. TT and MZ-CRC-1 tumor cell lines were cultured in F12 Nutrient Mixture (Gibco) and DMEM GlutaMax (invitrogen), respectively, supplemented with 10% (v/v) FBS (PAA Laboratories) and 1% (v/v) penicillin/streptomycin, as previously described (20).

Transfection of hsa-miR-30a-5p expression vector in MZ-CRC-1 cell line and RNA isolation

To determine whether the microRNA hsa-miR-30a-5p regulated the abundance of CD133 mRNA in human MTC cell, MZ-CRC-1 cell line was transfected with either a hsa-miR-30a-5p expression vector or a control vector. Briefly, 50 000 MZ-CRC-1 cells were seeded per well on 6-well plates (Corning). Cells were transfected with 2.5µg LentimiRa-GFP-hsa-miR-30a-5p Vector (mh10337; ABM) or pLenti-III-mir-GFP Control Vector (m001, ABM), 10µl Lipofectamine 2000 (Invitrogen) and 300µl Opti-MEM medium (Gibco) per well, as indicated by the manufacturer's instructions and total RNA was isolated at 24, 48 and 72 hours post-transfection with 500µl of Ribozol (VWR Life Science), following the standard protocol to determine the mRNA abundance of CD133 encoding gene, *PROM1*.

Quantification of PROM1 gene and hsa-miR-30a-5p miRNA expression

To quantify PROM1/CD133 mRNA, 1 µg of total RNA was reverse transcribed using the Superscript II reaction mix (Invitrogen) with a pool of random primers and $oligo(dT)_{14}$. PROM1/CD133 mRNA was quantified using specific primers (Forward: 5'-ctcccatacttcttagtttc-3'; Reverse: 5'-cagggaatggattgttgga-3' and the indicated probe (#6) from the universal Human ProbeLibrary Set (Roche). Expression of PROM1/CD133 mRNA was calculated by the ΔΔCt method using the Ct value of B-actin (ACTB) (Forward: 5'-ccagaggcgtacagggatag -3'; Reverse: 5'-ccaaccgcgagaagatga-3') to normalize the data (36).

To confirm hsa-miR30a-5p expression increase upon cell lines transfection with LentimiRa-GFP-hsa-miR-30a-5p Vector, miRNA expression was quantified for both all assay conditions as follows: 10ng of total RNA was reverse transcribed using the miRCURY LNA™ Universal RT miR PCR System (Exiqon), cDNA was 1:40 diluted and quantitative PCR was performed using microRNA LNA™ PCR primer sets (Exiqon) and Exilent SYBR™ Green Master Mix (Exiqon), following the manufacturer's protocol. The miRNA hsa-miR-16-5p, with a proven constant expression across human MTCs (GSE72728), was quantified as above and used to normalize the expression data as previously described (20).

All quantitative PCRs were performed using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Negative controls were included in all PCR series and reactions were carried out in triplicate.

MTT assay

The effect of hsa-miR-30a-5p exogenous expression on the viability of MZ-CRC-1 cells was quantified as follows: 10 000 cells/well were seeded on a 96-well plate and 24 hours later cells were transfected with 100ng/well of LentimiRa-GFP-hsa-miR-30a-5p Vector (mh10337; ABM) or pLenti-III-mir-GFP Control Vector (m001, ABM) using 0.4 µl of Lipofectamine 2000 (Invitrogen) in 25 µl of Opti-MEM medium (Gibco) per well as indicated by manufacturer's protocol. At day 1, 2 and 3 post-transfection, 50µl of MTT assay (Roche) solution was added to each well and the plate was incubated for 3 hours. After adding 200µl of DMSO (Sigma) per well the plate was shaken for 5 minutes and the absorbance at 540nm was measured using an EnVision 2104 Multilabel Reader (Perkin Elmer). The experiment included 9 replicates of each condition and was repeated twice.

Statistical analysis

Statistical analyses were performed using SPSS, version 19.0 (IBM SPSS Statistics, Armonk, NY) and statistical software R version 3.6.1 (http://www.R-project.org/). We tested the association between categorical variables using the Fisher's exact test or Pearson's chi-squared test. Progression-free survival was defined as the time between the date of primary surgery and either the date of local recurrence or distant metastasis, or death. The Kaplan-Meier method was used to calculate time to progression, and the log-rank test was used to determine whether differences in the outcomes were statistically significant. Multivariate weighted Cox regression analysis was performed to evaluate the association of patient and tumor variables with disease prognosis using *coxphw* function in R (37). Univariate analysis of the association between each variable and time to progression was calculated and hazard ratios (HRs) and 95% confidence intervals (CIs) were generated.

Student's t-test was used to evaluate the differences between in vitro experimental conditions, which were performed in triplicate and repeated at least twice. Spearman's correlation analysis was performed to calculate the association between the expression of the *PROM1* gene and *PROM1* mRNA-targeting miRNAs. Graphical representations were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, www.graphpad.com). All tests were two-sided, and *P*-values < 0.05 were considered statistically significant.

Results

Immunohistochemical expression of PROM1/CD133 is associated with MTC tumor and patient clinicopathological features

Specimens of 72 primary tumors and 2 lymph node metastases from independent MTC patients were available for evaluation of CD133 expression (unglycosylated and glycosylated protein). Immunohistochemical (IHC) staining revealed that PROM1/CD133 is specifically localized in the cell membrane and cytoplasm (occasionally dot-like staining, Golgi) of tumor cells (Fig. 1A). Expression-specificity of CD133 in C-cell cancer cells was confirmed by IHC staining of calcitonin, a specific marker of C-cells, on consecutive tissue sections for a subset of CD133-positive tumors. As shown in Fig. 1B and 1C, CD133 expressing cells were also positive for calcitonin immunostaining. The rate of CD133-expressing MTCs was 51% (38/74) and no significant differences were found in calcitonin protein IHC staining or mRNA expression between CD133-positive and -negative tumors (Fig. 1B and Supplementary Fig. 1) (31). Peritumoral blood vessels were observed in 73 out of 74 tumors. Notably, while 94.6% (35/37) of CD133-positive MTCs showed infiltrating cancer cells or tumor emboli in blood vessels adjacent to the tumor, only 11.1% (4/36) of CD133-negative MTCs were positive for this feature (P value < 0.001) (Fig. 1C and Table 1). Clinicopathological characteristics of tumors and patients, and detailed information of CD133 staining are shown in Supplementary Table 1 (31).

As expected, around 80% of MTCs were sporadic (82.4%, 61/74) and 20% were hereditary (17.6%, 13/74). Fisher exact test for sporadic MTCs showed that PROM1/CD133 expression is associated with male gender ($P = 0.003$), larger tumor size ($P < 0.001$), presence of regional lymph node metastases at diagnosis ($P < 0.001$), presence of distant metastasis at diagnosis ($P = 0.007$), advanced American Joint Committee on Cancer (AJCC) prognostic stage ($P < 0.001$), and the presence of tumor emboli in peritumoral blood vessels ($P < 0.001$) (Table 1). Although only this variable showed a significant association with PROM1/CD133 expression ($P = 0.029$) in the hereditary MTC series (Table 1), probably in part due to the limited sample size, significant associations observed in the sporadic series were maintained when hereditary cases were added to the association analysis.

MTC genotype association with CD133 expression and clinicopathological features of the patients

The 13 hereditary MTCs harbored a RET mutation with an associated risk to develop an aggressive MTC according to the American Thyroid Association guidelines (1) (Supplementary Table 1) (31). Five cases fit in the moderate-risk category, which should not be aggressive by definition, and 8 in the high-risk category. As expected, the RET genotype high-risk category was correlated with earlier age of onset ($P = 0.032$). However, we found a significant association of moderate-risk genotype with the

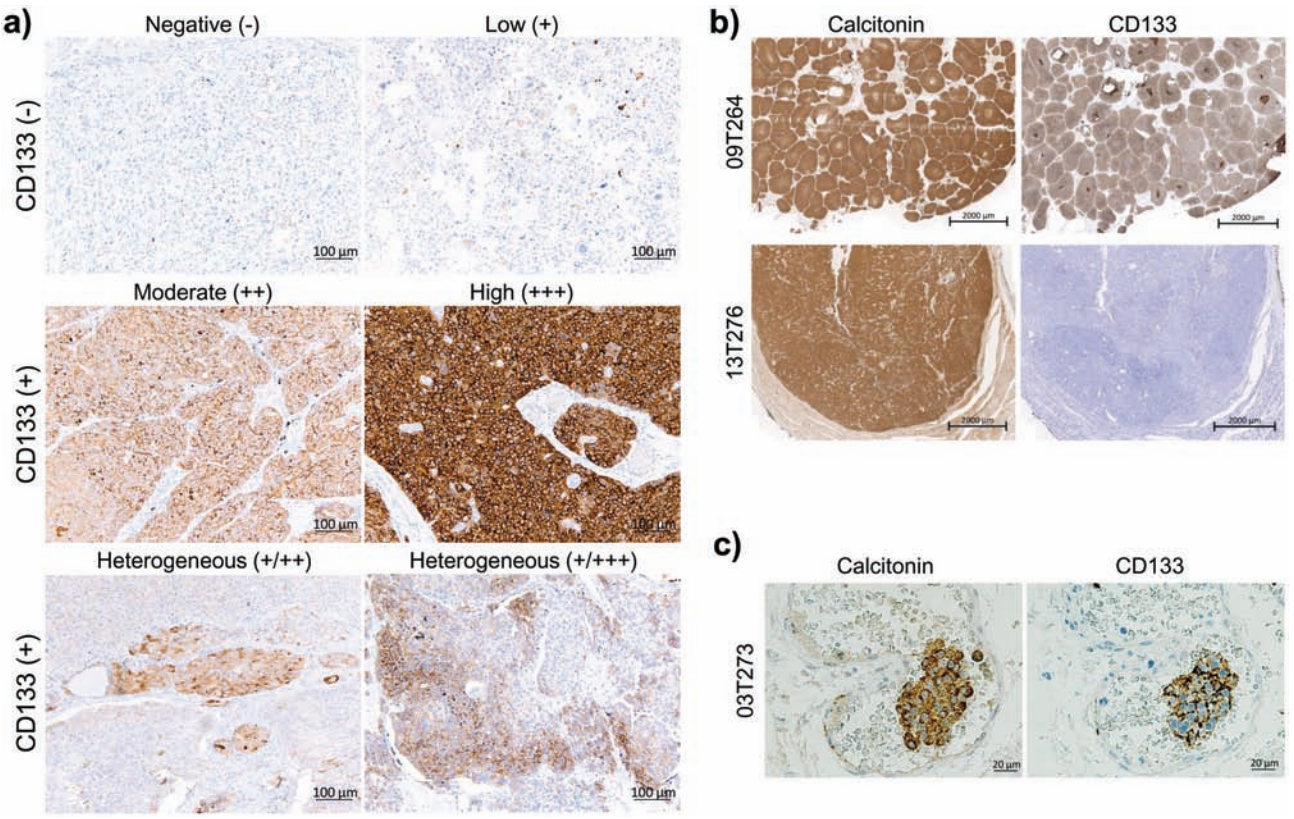


Figure 1. Expression of the stem cell marker CD133 in MTCs. a) Representative images of CD133 staining patterns. Tumors with moderate (++) (#14T44), high (+++) (#17T201), or heterogeneous moderate (#03T114)/high (#03T90) staining intensities were considered CD133 positive (+). Tumors with negative (-) (#13T276) or residual staining (low, +) (#14T42) were considered CD133 negative (-). b) Calcitonin immunohistochemical staining of representative CD133 (+) (#09T264) and CD133 (-) (#13T276) MTCs. c) Representative images of calcitonin and CD133 staining showing tumor cells infiltrating blood vessels adjacent to the tumor.

presence of distant metastases at diagnosis ($P = 0.033$) and a tendency to a significant association with the presence of regional lymph node metastases at diagnosis ($P = 0.081$). The association between RET genotype risk categories and CD133 expression did not reach the statistical significance (Table 2).

Sporadic MTCs were categorized into the 4 genetic groups established by Moura et al (38) to evaluate the correlation of tumor genotype with clinical variables and CD133 expression. Similar to other published series (13, 16, 38), the rates of these groups were 38.6% for RET^{M918T} (22/57), 28.1% for other RET mutations (16/57), 26.3% for RAS mutations (15/57), and 7.0% for the wild-type group (no RET or RAS mutations) (4/57). The analysis revealed a significant correlation of the genetic classes with CD133 expression ($P = 0.002$), the presence of tumor emboli in peritumoral blood vessels ($P < 0.001$), larger tumor size ($P = 0.001$), presence of regional lymph node metastasis at diagnosis ($P = 0.009$), and advanced AJCC prognostic stage ($P = 0.017$). Remarkably, these significant values seem to be mainly driven by the RAS-mutant class, as the RAS-MTCs showed the lowest frequency rates of many of these adverse features. On the opposite, RET^{M918T} MTCs showed the highest frequency rates for variables such as large tumor size and presence of lymph nodes metastases at diagnosis.

PROM1/CD133 expression as a predictor of disease progression

We have follow-up information for 39 of the 74 patients; 18 were disease-free after the primary surgery and until the last follow-up date (median = 7.5 years; range, 1-16 years), 20 patients developed local recurrence, distant metastasis or died due to the disease during the follow-up (median = 6.5 years; range, 1-18 years) and 1 patient died within the first year of follow-up. To explore the relationship of CD133 expression and prognosis, we performed disease progression-free survival analysis of this cohort. Kaplan-Meier curves and log-rank tests showed that CD133-positive patients had a significantly shorter time to disease progression than CD133-negative patients (log-rank test $P < 0.001$) (Fig. 2A). This difference stayed significant even when only RET-mutant cases were included

Table 1. Association Analysis of CD133 Protein Expression with MTC Clinicopathological Features

		Sporadic MTC (n = 61)					Hereditary MTC (n = 13)					Sporadic & Hereditary MTC (n = 74)				
		CD133 expression				Fisher's exact test	CD133 expression				Fisher's exact test	CD133 expression				Fisher's exact test
Features	Categories	Negative		Positive		*P* value	Negative		Positive		*P* value	Negative		Positive		*P* value
Age																
	<45	45.0%	(9/20)	55.0%	(11/20)	1000	75.0%	(6/8)	25.0%	(2/8)	0.103	53.6%	(15/28)	46.4%	(13/28)	0.479
	≥45	47.5%	(19/40)	52.5%	(21/40)		20.0%	(1/5)	80.0%	(4/5)		44.4%	(20/45)	55.6%	(25/45)	
Gender																
	Female	63.2%	(24/38)	36.8%	(14/38)	**0.003**	71.4%	(5/7)	28.6%	(2/7)	0.286	64.4%	(29/45)	35.6%	(16/45)	**0.001**
	Male	21.7%	(5/23)	78.3%	(18/23)		33.3%	(2/6)	66.7%	(4/6)		24.1%	(7/29)	75.9%	(22/29)	
Primary tumor size																
	T1-T2	72.7%	(24/33)	27.3%	(9/33)	**<0.001**	60.0%	(3/5)	40.0%	(2/5)	1.000	71.1%	(27/38)	28.9%	(11/38)	**0.001**
	T3-T4	14.3%	(2/14)	85.7%	(12/14)		40.0%	(2/5)	60.0%	(3/5)		21.1%	(4/19)	78.9%	(15/19)	
Regional Lymph node metastases at Dx																
	No	81.0%	(17/21)	19.0%	(4/21)	**<0.001**	50.0%	(2/4)	50.0%	(2/4)	1.000	76.0%	(19/25)	24.0%	(6/25)	**<0.001**
	Yes	21.9%	(7/32)	78.1%	(25/32)		50.0%	(4/8)	50.0%	(4/8)		27.5%	(11/40)	72.5%	(29/40)	
Distant metastases at Dx																
	No	62.5%	(20/32)	37.5%	(12/32)	**0.007**	57.1%	(4/7)	42.9%	(3/7)	1.000	61.5%	(24/39)	38.5%	(15/39)	**0.009**
	Yes	0.0%	(0/6)	100.0%	(6/6)		33.3%	(1/3)	66.7%	(2/3)		11.1%	(1/9)	88.9%	(8/9)	
AJCC (prognostic groups)																
	I-III	78.3%	(18/23)	21.7%	(5/23)	**<0.001**	50.0%	(2/4)	50.0%	(2/4)	1.000	74.1%	(20/27)	25.9%	(7/27)	**0.001**
	IV	13.3%	(2/15)	86.7%	(13/15)		50.0%	(3/6)	50.0%	(3/6)		23.8%	(5/21)	76.2%	(16/21)	
Tumor emboli in peritumoral blood vessels																
	No	96.3%	(26/27)	3.7%	(1/27)	**<0.001**	87.5%	(6/7)	12.5%	(1/7)	**0.029**	94.1%	(32/34)	5.9%	(2/34)	**<0.001**
	Yes	9.1%	(3/33)	90.9%	(30/33)		16.7%	(1/6)	83.3%	(5/6)		10.3%	(4/39)	89.7%	(35/39)	

Significant *P* values (≤0.05) are highlighted in bold.

Table 2. Association Analysis of Tumor Genotype Categories With MTC Clinicopathological Features and CD133 Protein Expression

		Sporadic MTCs										Hereditary MTCs				
		Sporadic MTC genotype groups[a] (n = 57)								Pearson chi-squared test		RETmut risk categories[b] (n = 13)				Fisher exact test
Features	Categories	RET^{M918T} (n = 22)		RET^{Other} (n = 16)		RAS (n = 15)		WT (n = 4)		χ^2	*P* value	Moderate (n = 5)		High (n = 8)		*P* value
Age																
	<45	40.9%	(9/22)	25.0%	(4/16)	20.0%	(3/15)	66.7%	(2/3)	3803	0.284	20.0%	(1/5)	87.5%	(7/8)	**0.032**
	≥45	59.1%	(13/22)	75.0%	(12/16)	80.0%	(12/15)	33.3%	(1/3)			80.0%	(4/5)	12.5%	(1/8)	
Gender																
	Female	50.0%	(11/22)	62.5%	(10/16)	73.3%	(11/15)	100.0%	(4/4)	4641	0.200	60.0%	(3/5)	50.0%	(4/8)	1
	Male	50.0%	(11/22)	37.5%	(6/16)	26.7%	(4/15)	0.0%	(0/4)			40.0%	(2/5)	50.0%	(4/8)	
Primary tumor size																
	T1-2	31.3%	(5/16)	83.3%	(10/12)	92.3%	(12/13)	100.0%	(2/2)	15 551	**0.001**	25.0%	(1/4)	66.7%	(4/6)	0.524
	T3-4	68.8%	(11/16)	16.7%	(2/12)	7.7%	(1/13)	0.0%	(0/2)			75.0%	(3/4)	33.3%	(2/6)	
Regional lymph node metastases at Dx																
	No	10.5%	(2/19)	46.7%	(7/15)	66.7%	(8/12)	66.7%	(2/3)	11 695	**0.009**	0.0%	(0/5)	57.1%	(4/7)	0.081
	Yes	89.5%	(17/19)	53.3%	(8/15)	33.3%	(4/12)	33.3%	(1/3)			100.0%	(5/5)	42.9%	(3/7)	
Distant metastases at Dx																
	No	72.7%	(8/11)	70.0%	(7/10)	100.0%	(11/11)	100.0%	(3/3)	4855	0.183	25.0%	(1/4)	100.0%	(6/6)	**0.033**
	Yes	27.3%	(3/11)	30.0%	(3/10)	0.0%	(0/11)	0.0%	(0/3)			75.0%	(3/4)[c]	0.0%	(0/6)	
AJCC (prognostic groups)																
	I-III	27.3%	(3/11)	50.0%	(6/12)	90.0%	(9/10)	100.0%	(2/2)	10 166	**0.017**	20.0%	(1/5)	60.0%	(3/5)	0.524
	IV	72.7%	(8/11)	50.0%	(6/12)	10.0%	(1/10)	0.0%	(0/2)			80.0%	(4/5)	40.0%	(2/5)	
Tumor emboli in peritumoral blood vessels																
	No	13.6%	(3/22)	31.3%	(5/16)	93.3%	(14/15)	66.7%	(2/3)	24 851	**<0.001**	40.0%	(2/5)	62.5%	(5/8)	0.592
	Yes	86.4%	(19/22)	68.8%	(11/16)	6.7%	(1/15)	33.3%	(1/3)			60.0%	(3/5)	37.5%	(3/8)	
CD133 expression																
	Negative	22.7%	(5/22)	37.5%	(6/16)	86.7%	(13/15)	50.0%	(2/4)	15.291	**0.002**	20.0%	(1/5)	75.0%	(6/8)	0.103
	Positive	77.3%	(17/22)	62.5%	(10/16)	13.3%	(2/15)	50.0%	(2/4)			80.0%	(4/5)	25.0%	(2/8)	

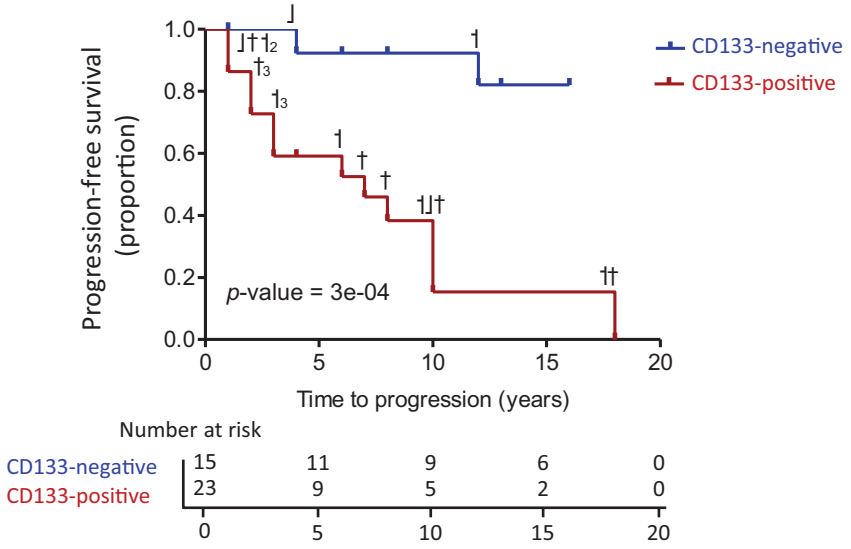
Significant *P* values (≤0.05) are highlighted in bold.

[a]Moura et al (2015)

[b]Wells et al (2015)

[c]2 cases are RET^{C618F} mutant and 1 is RET^{S891A} mutant.

a)



b)

	Univariate analysis (PFS)				Multivariate analysis (PFS)			
	n	Event (n)	HR (95% CI)	*p*-value	n	Event (n)	HR (95% CI)	*p*-value
CD133 expression (+)	38	20	24.259 (4.19-140.4)	**0.0004**	37	20	16.619 (3.04-90.79)	0.0012
Tumor emboli in peritumoral blood vessels (+)	37	20	4.675 (1.53-14.25)	**0.0067**	37	20	2.031 (1.19-3.47)	0.0095
Age (≥45 years)	38	20	2.725 (1.02-7,271)	0.0453				
Gender (male)	38	20	2.744 (1.25-6.01)	0.0116				
AJCC (III-IV)	30	14	6.030 (1.49-24.38)	0.0117				

PFS: Progression-free survival; n: number of MTCs included in the analysis; HR: Hazard ratio; CI: Confidence interval.

Univariate analyses with p-values<0.01 are highlighted in bold.

Figure 2. a) Progression-free survival curves of MTC patients with available follow-up data (n = 39). Patients were dichotomized into 2 groups according to CD133 expression. ↿: local disease progression; ⇂: distal disease progression; †: exitus. Numbers to the right of the symbols indicate the number of patients with that event when different than 1. b) Results of univariate and multivariate weighted Cox regression analysis to evaluate risk factors for hazard of progressive disease. AJCC prognostic groups were dichotomized into stages I-II and III-IV. Univariate analyses with *P* values < 0.01 are highlighted in bold. Abbreviations: CI, confidence interval; HR, hazard ratio; n: Number of MTCs included in the analysis; PFS: Progression-free survival.

in the analysis (log-rank test $P < 0.003$) (Supplementary Fig. 2) (31). Next, since the clinical factors age, gender, AJCC prognostic group, and presence of tumor emboli in peritumoral blood vessels have been previously associated with poor prognosis in MTC, and with CD133 expression (Table 1), we decided to examine which of these covariates have the most impact on progression-free survival. Univariate Cox regression models revealed that CD133 expression (HR = 24.26; 95% CI, 4.19-140.40; $P < 0.001$) and presence of tumor emboli in peritumoral blood vessels (HR = 4.68; 95% CI, 1.53-14.25; $P = 0.007$) were the covariates with the strongest association with progression-free survival ($P < 0.01$) (Fig. 2B). Multivariate weighted Cox regression analysis including these 2 covariates showed that both CD133 expression and presence of tumor emboli in peritumoral blood vessels were independent risk factors ($P = 0.001$ and $P = 0.010$, respectively), with CD133 impact on prognosis being the highest (HR = 15.6) (Fig. 2B). Remarkably, these associations remained significant in a multivariate model adjusted for age, gender, and AJCC prognostic stage (Supplementary Table 2) (31).

Mechanisms of regulation of PROM1/CD133 expression in MTC cells

In silico analysis identified the microRNA hsa-miR-30a-5p as a putative regulator of Prom1/Cd133 mRNA abundance (Fig. 3A). Hsa-miR-30a-5p downregulation has been previously identified as a molecular hallmark of clinically aggressive MTCs (20). Therefore, we decided to evaluate whether

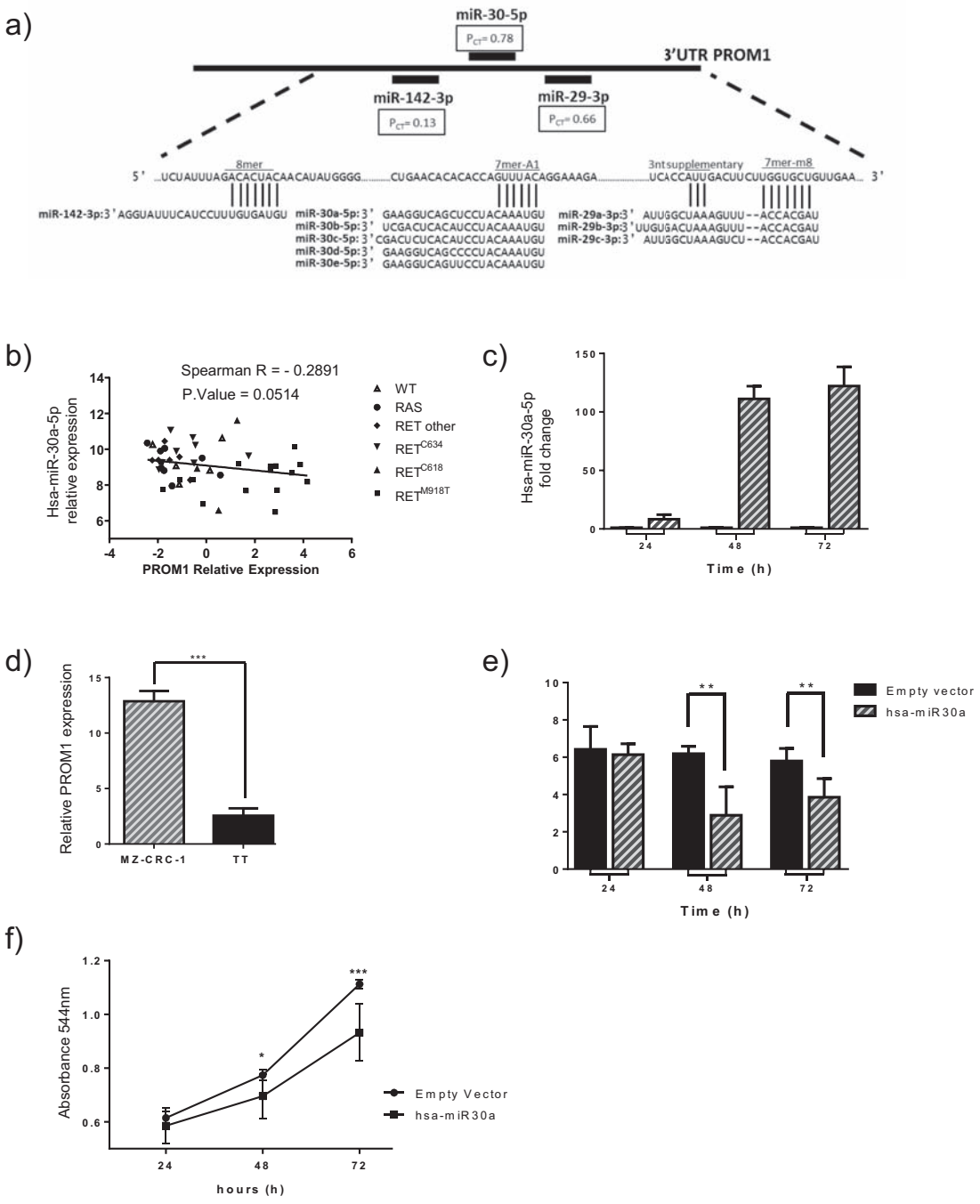


Figure 3. Regulation of expression of the CD133-encoding gene *PROM1*. a) Schematic representation of *PROM1* 3′ UTR conserved miRNA-targeting sites (Targetscan 7.2 database). b) Spearman correlation analysis between hsa-miR30a-5p and *PROM1* gene expression in MTCs. c) Hsa-miR30a-5p expression fold-change in MZ-CRC-1 cell line transfected with miR30a-5p expression vector relative to control condition at indicated post-transfection times. d) Relative expression of *PROM1* gene in human MTC cell lines MZ-CRC-1 (RET^{M918T}) and TT (RET^{C634Y}). e) Relative PROM1 expression in MZ-CRC-1 cell line transfected with either the miR30a-5p expression vector or a control vector at indicated post-transfection times. f) MTT assay plot for MZ-CRC-1 cell line transfected with either a miR30a-5p expression vector or a control vector. Cell viability of both conditions was determined at indicated post-transfection times. All in vitro assays were performed at least in triplicate and repeated twice. T-test significant *P* values are shown (* 0.05-0.01, ** 0.01-0.001, ***<0.001).

hsa-miR-30a-5p regulates *PROM1* expression in MTC. We first analyzed the correlation between the miRNA and gene expression in primary MTCs using the publicly available data of the miRNome (GSE72728) (20) and transcriptome (GSE32662) (23) for a series of 31 MTCs that we extended to 46 (unpublished data). Spearman correlation analysis showed a tendency to a significant inverse correlation between hsa-miR-30a-5p and *PROM1* expression (Spearman $R = -0.289$; $P = 0.0514$) (Fig. 3B). Next, we expressed this miRNA ectopically in the human MTC cell line

MZ-CRC-1 (Fig. 3C), which harbors the RET^{M918T} mutation and has a detectable expression of *PROM1* (Fig. 3D). Of note, we found that PROM1 mRNA abundance showed a significant reduction upon hsa-miR30a-5p expression (30%-50%) at 48 to 72 hours postinduction ($P = 0.01$) (Fig. 3E). In addition, similar to previously published results with knockdown assays for *PROM1* expression (23), induction of hsa-miR-30a expression significantly reduced viability of MZ-CRC-1 cells compared with a nontargeting control condition ($P < 0.01$, 72-hour postexpression induction) (Fig. 3F).

Discussion

MTC is considered the differentiated thyroid tumor with the worst clinical outcome (1). Identification of molecular factors that anticipate the clinical course of the disease is critical to improve MTC management and patient survival rates. In this sense, *RET* genotype is the biomarker currently used to stratify hereditary disease patients according to the risk to develop an aggressive MTC. However, similar to recent reports (8, 9), in the present study we found that high-risk category was only associated with an earlier age of onset but not with other adverse clinical variables when compared with moderate-risk MTCs. In fact, the moderate-risk category showed a higher prevalence of local and/or distant metastases at diagnosis than the high-risk category, which was also observed in a large hereditary MTC series (8), although underlying reasons are not understood. In sporadic MTCs, we found that the genetic class RET^{M918T} was the sMTC entity with significantly highest frequency of cases with adverse features such as larger tumor size and presence of lymph nodes at diagnosis, which has been historically reported and recently validated in a large series of MTCs (39). On the other side, *RAS*-mutant MTCs seem to be the least virulent entity, as this genetic class had the lowest rates of local and distant metastasis at diagnosis and was almost negative for vascular invasion or presence of tumor emboli in peritumoral blood vessels, which is a feature of poor outcome MTCs (40). This is in accordance with earlier publications (15, 16, 38) that systematically have shown that the *RAS*-mutant class compiles less aggressive entities. Notably, the association of tumor genotype with disease prognosis has constraints, and while not all RET^{M918T} sMTCs are clinically aggressive, many sMTCs with other RET mutations are highly aggressive.

Therefore, considering the aforementioned prognostic limitations of tumor genetics, there is a clinical necessity of identifying molecular predictors for progressive disease. In this regard, high PROM1/CD133 expression has been previously associated with aggressive MTCs (23) and reduced survival (24), but its value as an independent risk factor of prognosis has not been established. Here we found that CD133 expression was associated with adverse clinicopathological features previously reported by Bi and collaborators (24) and also with male gender, which emerged as a significant covariate of progression-free survival in the univariate analysis (27, 41, 42). Notably, multivariate analysis including this and other clinical factors previously associated with MTC prognosis such as AJCC prognostic stage, revealed that CD133 expression was the covariate with the highest impact on progression-free survival and emerged as an independent prognostic risk factor. Although the statistical power was limited by series substratification for nonbinary variables, the results consistently show that CD133 is a promising biomarker of prognosis in MTCs. Furthermore, CD133 status dichotomized RET-mutant MTCs according to the time to disease progression, supporting that CD133 prognostic value is independent of tumor driver gene.

With regard to CD133 function, it is widely accepted that CD133 expression is a marker of cancer stem cells (43) and it has been associated with a more undifferentiated phenotype in MTC cells in in vitro assays (27). However, MTC is a well differentiated thyroid cancer that retains the primary function of normal parafollicular cells, the synthesis and secretion of the peptide hormone calcitonin. Therefore, high levels of calcitonin in serum and positive calcitonin immunoreactivity in tumors are universal biomarkers of the disease and used in the clinical practice for the diagnosis and follow-up of MTC patients (1). Notably, although rare, there are extremely aggressive and undifferentiated MTCs that lose the ability to produce or secrete calcitonin, showing normal or only mildly increased serum calcitonin (44, 45) and a reduced immunoreactivity of calcitonin in the tumors (1). Considering all this, we decided to explore whether calcitonin expression showed any reduction in CD133-positive compared to CD133-negative tumors, as a sign of some degree of undifferentiation. We did not observe any significant differences between CD133-positive and -negative tumors, either by calcitonin immunohistochemical analysis, or by gene expression quantification, suggesting that CD133 is not a regulator of stemness in MTCs. In this sense, CD133 expression has also been identified in differentiated cells supporting the hypothesis that CD133 has other unknown functions (46). Future studies with appropriate models are required to clarify the functionality of CD133 expression in MTC.

Regarding the regulation of CD133 expression in cancer, several miRNAs (hsa-miR-142-3p, hsa-miR-29-3p, hsa-miR-135a/b, hsa-miR-1915, hsa-miR-377, and hsa-miR-487b) have been described to inhibit CD133 expression in tumor cells of multiple lineages (28-30, 47-49), pointing at miRNA-deregulation as a key mechanism driving increased

PROM1/CD133 expression in cancer. Specifically in MTC, we previously found that the miRNA hsa-miR-30a-5p was highly expressed in aggressive MTCs (20) and, intriguingly, this miRNA is 1 of the 3 miRNAs predicted to target the 3′ UTR of *PROM1*. Here, we found that hsa-miR-30a-5p expression in MTC cells was correlated with a reduction of PROM1 mRNA abundance and cell viability. The findings fit with the earlier described role of PROM1/CD133 in cell survival (23) and point to hsa-miR-30a-5p expression as a negative regulator of CD133 expression in MTCs.

Overall, our study reveals CD133 expression as a risk factor of disease progression and identifies a regulatory mechanism of PROM1/CD133 expression in MTC cells. Furthermore, we found that most CD133-positive MTCs have cancer cells inside blood vessels, suggesting that CD133-positive MTC cells may be present in the bloodstream of these patients. Studies aimed at detecting tumor cells expressing CD133 in the blood of MTC patients may support this marker as a potential predictor of disease virulence, as described in patients with other solid tumors (50-52), and improve the surgical decision-making process and follow-up of MTC disease.

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Disclosure Summary: All authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References. Extended Data Sets and Supplemental Materials are available as cited (31).

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