Increased Global DNA Hypomethylation in Distant Metastatic and Dedifferentiated Thyroid Cancer

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Context: Global DNA hypomethylation is a major event for the development and progression of cancer, although the significance in thyroid cancer remains unclear. Therefore, we aimed to investigate its role in thyroid cancer progression and its potential as a prognostic marker.

Methods: Global hypomethylation of Alu repeats was used as a surrogate marker for DNA global hypomethylation, and was assessed using the Quantification of Unmethylated Alu technique. Mutations in *BRAF* and *RAS* were determined by Sanger sequencing.

Results: Ninety primary thyroid tumors were included [28 low-risk differentiated thyroid cancer (DTC), 13 pediatric DTC, 33 distant metastatic DTC, 7 poorly differentiated thyroid cancer (PDTC), and 9 anaplastic thyroid cancer (ATC)], as well as 24 distant metastases and 20 normal thyroid tissues. An increasing hypomethylation was found for distant metastatic DTC [median, 4.0; interquartile range (IQR), 3.1 to 6.2] and PDTC/ATC (median, 9.3; IQR, 7.0 to 12.1) as compared with normal thyroid tissue (median, 2.75; IQR, 2.30 to 3.15), whereas low-risk and pediatric DTC were not affected by hypomethylation. Alu hypomethylation was similar between distant metastases and matched primary tumors. Within distant metastatic DTC, Alu hypomethylation was increased in *BRAF* vs *RAS* mutated tumors. Kaplan–Meier and Cox regression analyses showed that thyroid cancer–related and all-cause mortality were associated with tumor hypomethylation, but this association was lost after adjustment for thyroid cancer risk category.

Conclusion: Distant metastatic DTC, PDTC, and ATC were increasingly affected by global Alu hypomethylation, suggesting that this epigenetic entity may be involved in thyroid cancer progression and dedifferentiation. (***J Clin Endocrinol Metab*** **103: 397–406, 2018)**

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Abbreviations: ATC, anaplastic thyroid cancer; CI, confidence interval; DTC, differentiated thyroid cancer; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; IQR, interquartile range; LINE-1, long-interspersed nuclear element-1; PDTC, poorly differentiated thyroid cancer; PUMA, percentage of unmethylated Alu; qPCR, quantitative polymerase chain reaction; QUAlu, Quantification of Unmethylated Alu; WT, wild-type.

Thyroid cancer is an increasingly common malignancy, which is especially true for differentiated thyroid cancer (DTC) (1). In particular, patients with low-risk DTC and pediatric DTC have an excellent prognosis, although the latter generally present with rather extensive disease (2, 3). However, for patients with DTC who develop distant metastases or radioiodine-refractory disease, the prognosis is poor (4), which is the case as well for patients with poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid carcinoma (ATC) (5). When initial treatment fails in these high-risk thyroid cancer patients, an effective treatment is currently lacking. The key for the future development of treatments probably lies in an improved understanding of the molecular events in tumors of DTC patients with distant metastatic disease, as well as in patients with PDTC and ATC.

Genetic and epigenetic alterations are critical players in cancer development and progression (6). Several genetic alterations that affect the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT pathways have been found in DTC (7, 8). Of these, mutations in *BRAF* and *RAS* and *RET/PTC* and *PAX8/PPARG* rearrangements are the most common for DTC. Furthermore, increasing amounts of data are becoming available about epigenetic modifications in thyroid cancer, especially DNA methylation (9–12). DNA methylation is one of the most well-characterized epigenetic entities that consists of the addition of a methyl group to a cytosine. In mammals, this mainly occurs in cytosines that precede a guanine (a so-called CpG site) and constitutes an inactive mark associated with transcriptional inactivation and repressed chromatin. In cancer, two main alterations in DNA methylation have been distinguished (13). On the one hand, it is common to find locus-specific hypermethylation that mainly affects regulatory elements such as promoters, which can lead to silencing of tumor suppressor genes or genes that are important for cellular function (for example DNA repair and apoptosis) (14). On the other hand, tumoral cells often show a global DNA hypomethylation, which affects extensive domains of the genome and promotes genomic instability. Interestingly, a growing body of evidence shows an association between loss of DNA methylation and the early stages of tumorigenesis or tumor progression, and therefore it has been proposed as a cancer biomarker (15–17).

Especially repetitive DNA elements such as Alu and long-interspersed nuclear element-1 (LINE-1) repeats are altered by global DNA hypomethylation (18). Nearly half of the human genome is comprised of repetitive sequences, with Alu repeats being the most abundant (19). Alu elements are primate-specific transposons (*i.e.*, they can move within the genome) and make up almost 11% of the genomic mass (20). Approximately 25% of all human CpG sites are located within Alu repeats and most of them are methylated. However, a fraction remains unmethylated and this proportion is increased in cancer (21). Alu methylation levels have been shown to correlate well with global DNA methylation and could therefore be used as a surrogate marker for global hypomethylation (22). In this regard, several studies found a global Alu hypomethylation in different cancer types (16, 23–25).

Because the role of global DNA hypomethylation remains unclear in progressive thyroid cancer, we sought to investigate whether it is increased in primary tumors of patients with low-risk and distant metastatic DTC, pediatric DTC, and PDTC and ATC, using the recently developed Quantification of Unmethylated Alu (QUAlu) technique (25). Furthermore, we aimed to assess whether global Alu hypomethylation is altered in distant metastases as compared with their primary tumors, and whether it can act as a marker to differentiate between patients at risk for thyroid cancer–related and all-cause mortality.

Materials and Methods

Patients and samples

We analyzed tumor tissue of patients with low-risk DTC [defined as patients with well-differentiated papillary thyroid cancer (PTC) or follicular thyroid cancer (FTC) with tumor stage T1–2, Nx–N1, M0, who were disease-free after initial treatment and remained disease-free during a follow-up of at least 5 years] and distant metastatic DTC (defined as PTC or FTC patients with distant metastases at presentation or during follow-up). Furthermore, we included tumor tissues from patients with PDTC, ATC, and childhood-onset PTC. The study was accepted by the Ethics Committees of the participating centers (Supplemental Methods). All patients who were still alive during sample recruitment provided written informed consent for use of their thyroid tissue.

Primary tumors (n = 90) and, if available, distant metastases (n = 24, mostly from bone or lung, paired with 19 primary tumors) were obtained after examination by an experienced pathologist. Furthermore, several paired normal thyroid tissues (n = 20) were analyzed. Normal tissue was defined as nontumoral thyroid tissue adjacent to tumoral tissue, as indicated by the pathologist. Formalin-fixed paraffin-embedded (FFPE) tissues were used for patients with DTC. For patients with ATC and PDTC, fresh-frozen tissues were considered as well because FFPE tissues were scarce. Additionally, we included 10 fresh-frozen nonpaired normal tissues to compare with FFPE normal tissues. For each FFPE sample, 10 nonstained slices of 10-μm thickness (containing at least 80% tumoral cells to minimize the effect of contamination by normal cells, *i.e.*, underestimation of hypomethylation) were cut from the paraffin blocks. Thereafter, genomic DNA was extracted (Supplemental Methods).

Cell lines

See Supplemental Methods.

QUAlu

As previously described in detail (25), the QUAlu technique allows the relative quantification of unmethylated Alu repeats. It relies on the amplification of Alu elements with an unmethylated CpG site within the consensus Alu sequence AACCCGG. First, DNA was digested in separate tubes (both containing 5 ng of DNA) using the methylation-sensitive and -insensitive restriction enzymes *Hpa*II and *Msp*I, respectively, whose restriction site is C/CGG. *Hpa*II solely cuts unmethylated CpG sites, whereas *Msp*I cuts these sites irrespective of methylation status. Both enzymes leave an identical sticky end, to which a synthetic adaptor was subsequently ligated. The last step consisted of amplification of the *Hpa*II and *Msp*I digested-ligated DNA (1:20 diluted) by quantitative polymerase chain reaction (qPCR) using a primer homologous to the adapter and a primer specific for the Alu consensus sequence. Furthermore, we performed a specific qPCR to amplify L1PA (a LINE-1 subfamily), as an internal control to normalize DNA input. This finally allowed the calculation of the percentage of unmethylated Alu (PUMA) elements for each sample.

Mutation analyses

Primary adult tumors were analyzed by Sanger sequencing for the *BRAF* mutation at codon 600 in exon 15, and for *H-*, *N-*, and *K-RAS* mutations at codons 12 and 13 in exon 2 and codon 61 in exon 3 as follows: PTC tissues were screened for *BRAF* mutation, whereas follicular variant PTCs with no *BRAF* mutation and FTC samples were screened for *RAS* mutations. ATC and PDTC samples were screened for both *BRAF* and *RAS* genes. Primers are listed in Supplemental Table 1.

Study definitions

For all patients, clinical data were obtained, that is, baseline characteristics (sex, age at diagnosis, tumor histology, tumor–node–metastasis classification), treatment characteristics (surgery, radioiodine treatments and dose, use of tyrosine kinase inhibitors), and survival data. Follow-up time was defined as the time between the date of thyroid cancer diagnosis and the date of the last follow-up record or death.

Statistical analysis

Data were presented as number (percentage), median [interquartile range (IQR)], or mean ± standard deviation, as appropriate. The PUMA was calculated [as previously described (25)] as the ratio between quantifiable unmethylated Alu elements (measured in *Hpa*II digested DNA and normalized by L1PA) and total amount of amplifiable Alu elements (measured in *Msp*I digested DNA and normalized by L1PA). Permutation tests were performed using R and the qpcR package (v1.4-0) (26) to obtain the final PUMA. PUMA was compared between normal thyroid tissues and several thyroid cancer risk categories (low-risk DTC, pediatric PTC, distant metastatic DTC, and PDTC/ATC) using the Mann–Whitney *U* test. The Wilcoxon signed rank test was applied to test differences between PUMAs of paired thyroid tissues (normal vs primary tumor and primary tumor vs distant metastasis). A thyroid tumor was considered hypomethylated when the PUMA was above the 99th percentile of PUMA of normal tissues (25), which was 4.3%. The association between PUMA and mutational status was tested using the Mann–Whitney *U* test. Correlation analyses were performed using a Spearman Rho. Kaplan–Meier and unadjusted and adjusted Cox regression analyses were performed to assess the relationship between PUMA and thyroid cancer–related and all-cause mortality.

A P value < 0.05 was considered statistically significant. Analyses were performed using SPSS (version 22.0) and R (version 3.2.2).

Results

Patient characteristics

We included 90 patients with thyroid cancer for this study. A total of 28 patients had low-risk DTC, 33 had distant metastatic DTC (30 with synchronous distant metastases and 3 with metachronous distant metastases that occurred at least 6 months after diagnosis), 7 had PDTC, 9 had ATC, and 13 pediatric patients were diagnosed with PTC. See Table 1 for baseline and tumor characteristics and Supplemental Table 2 for an overview of the treatments administered and the survival data.

Technical evaluation of QUAlu in FFPE tissues

The QUAlu technique has previously been applied to a wide range of different clinical biospecimens (25), but it has not been extensively examined in FFPE tissues. Using different starting amounts of FFPE tissue–derived DNA (ranging from 0.3 to 80 ng), we obtained an excellent linear response ($R^2 > 0.95$) in quantification cycle values (or number of PCR cycles needed to exceed the background level, *i.e.*, the lower the quantification cycle value, the more DNA is present) (27) in the different qPCRs (Supplemental Fig. 1). This indicates that the QUAlu technique can be applied to a wide variety of DNA input amounts, including very low quantities. PUMA was equal in DNA from normal thyroid tissues derived from fresh-frozen (n = 10) and FFPE (n = 20) tissues [median (IQR) PUMA, 2.9 (2.7 to 3.2) and 2.8 (2.3 to 3.2], respectively, $P = 0.307$]. Moreover, PUMA for paired FFPE and fresh-frozen samples from normal thyroid and primary thyroid tumor tissues was comparable (Supplemental Table 3). Additionally, we analyzed the colon cancer cell line HCT116 using DNA from fresh-frozen cells and from a FFPE cell block and found similar PUMAs.

Global DNA hypomethylation of thyroid tumors

The QUAlu technique was applied to a series of normal thyroid tissues, primary thyroid carcinomas, and distant metastases (Fig. 1; Table 2). The PUMAs of FFPE normal tissues (n = 20) were homogeneous (median, 2.8; IQR, 2.3 to 3.2), and these were similar to the PUMAs of primary low-risk DTC tumors (median, 2.9; IQR, 2.6 to 3.5; $P = 0.336$). In contrast, primary tumors of patients with distant metastatic DTC had a significantly higher PUMA as compared with normal tissues (median, 4.0; IQR, 3.1 to 6.2), as well as patients with PDTC/ATC

Table 1. Baseline and Tumor Characteristics

Parameter	Low-Risk DTC	M1 DTC	PDTC/ATC	Pediatric PTC
N	28	33	16	13
Age, y, mean ± SD	45.1 ± 13.6	59.5 ± 14.2	67.8 ± 15.4	14.8 ± 2.8
Female sex, n (%)	23 (82.1)	25 (75.8)	10 (62.5)	11 (84.6)
Histology, n (%)				
PTC	20 (71.4)	20 (60.6)		13 (100)
FTC	8 (28.6)	13 (39.4)		
ATC			9 (56.3)	
PDTC			7 (43.8)	
TNM classification, n (%)				
T stage				
T1	10 (35.7)	5 (15.2)	0	4 (30.8)
T2	18 (64.3)	6 (18.2)	2 (12.5)	2 (15.4)
T3	0	10 (30.3)	4 (25)	5 (38.5)
T4	0	12 (36.4)	10 (62.5)	2 (15.4)
N stage				
Nx–N0	19 (67.9)	16 (48.5)	5 (31.3)	6 (46.2)
N1	9 (32.1)	17 (51.5)	11 (68.8)	7 (53.8)
M stage				
Mx–M0	28 (100)	0	7 (43.8)	10 (76.9)
M1	0	33 (100)	9 (56.3)	3 (23.1)
M1 site				
Bone	0	9 (27.3)	0	0
Lung	0	14 (42.4)	1 (11.1)	3 (100)
Multiple sites	0	9 (27.3)	2 (22.2)	0
Other	0	1 (3.0)	0	0
Unknown	0	0	6 (66.7)	0

M1 DTC indicates distant metastatic DTC.

Abbreviations: SD, standard deviation; TNM, tumor–node–metastasis.

(median, 9.3; IQR, 7.0 to 12.1) (both $P < 0.001$). In accordance, the PUMA was equal between normal thyroid and matched primary tumor tissues of low-risk DTC (n = 13, $P = 0.784$), whereas the PUMA of primary tumors of patients with distant metastatic DTC was higher as compared with the matched normal thyroid tissues (n = 7, $P = 0.018$) (Supplemental Fig. 2). The PUMA of pediatric PTC patients was a median of 3.2 (IQR, 2.9 to 3.6), which was not statistically different from the PUMA of normal thyroid tissue of adult patients ($P = 0.057$).

The percentages of hypomethylated tumors (*i.e.*, a PUMA above the 99th percentile of normal tissues) by thyroid cancer risk category are shown in Table 2. Tumors of patients with low-risk DTC and pediatric PTC were rarely hypomethylated, whereas distant metastatic DTCs were hypomethylated in 42% of cases. PDTCs and ATCs were highly hypomethylated in 94% of cases.

When available, we analyzed distant metastases as well (n = 24, matched with 19 patients; see Supplemental Table 4). The PUMA of distant metastases were similar to the PUMA of the matched primary thyroid tumor ($P = 0.344$) (Supplemental Fig. 3).

Furthermore, we quantified the PUMA of different thyroid cancer cell lines. All of them were highly hypomethylated in comparison with normal thyroid tissue (Supplemental Fig. 4).

Relationship between mutational status and PUMA

In Table 3 the *BRAF* and *RAS* mutation status is presented by histology and risk category (tumors from pediatric patients were not included). A total of 14 tumors were *BRAF* mutated, 11 tumors were *RAS* mutated, and 40 were wild-type (WT) for *BRAF* and *RAS*. The mutational status of one or both of these genes was unknown for 12 tumors. PUMA was similar among *BRAF* or *RAS* mutated and WT tumors ($P = 0.167$ and $P = 0.272$, respectively) (Table 3).

PUMA of primary tumors WT for both *BRAF* and *RAS* from distant metastatic DTC patients ($n = 14$) was a median of 4.2 (IQR, 2.9 to 6.5), as compared with 5.3 (4.5 to 8.1) for *BRAF* mutated (n = 4, $P = 0.277$) and 3.1 (2.4 to 3.5) for *RAS* mutated distant metastatic DTC (n = 6, $P = 0.051$). PUMA was higher in *BRAF* vs *RAS* mutated tumors ($P = 0.010$) (see Supplemental Fig. 5). In this sense, all BRAF mutated tumors were hypomethylated, whereas none of the *RAS* mutated tumors was hypomethylated. In all, 43% of distant metastatic DTC WT for both *BRAF* and *RAS* were hypomethylated. For low-risk DTC and for PDTC/ATC patients,

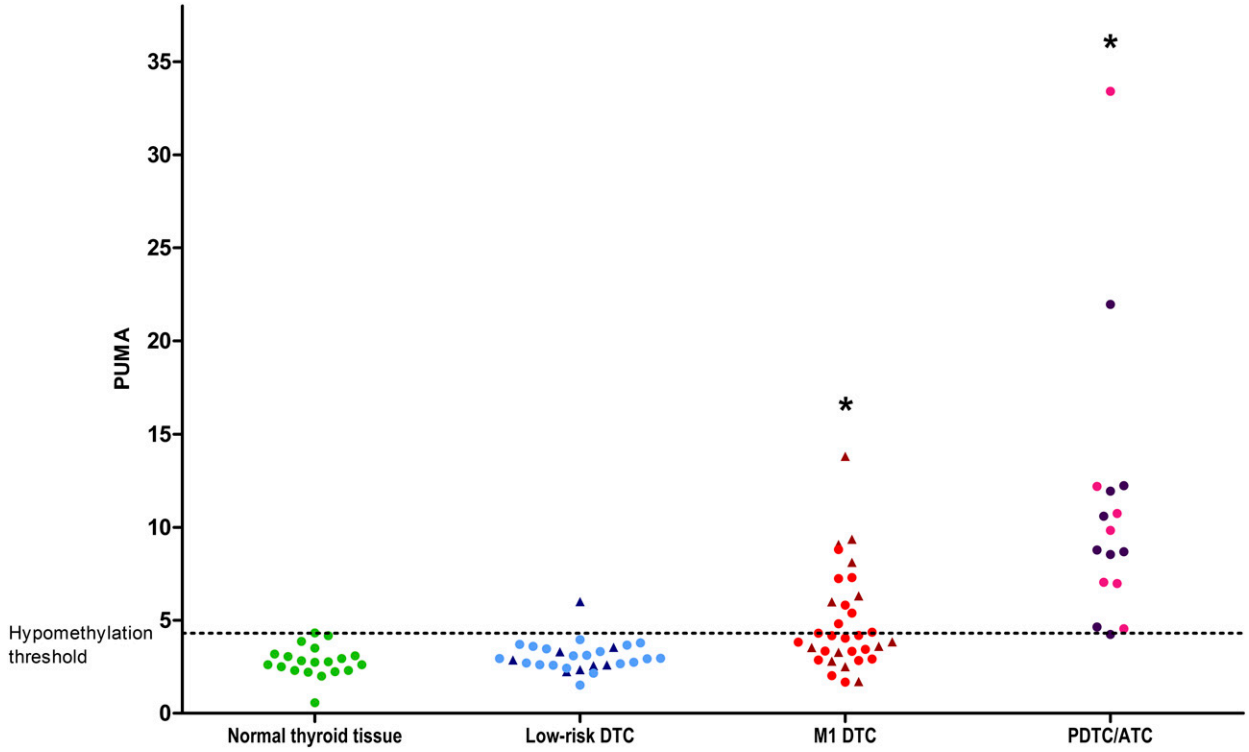

Figure 1. PUMA of normal thyroid tissues and (nonpediatric) primary thyroid cancers, represented by thyroid cancer risk categories. The darker triangle-shaped points within DTC categories represent patients with FTC; the lighter points represent PTC patients. Within the PDTC/ATC category, the darker and lighter points represent ATC and PDTC patients, respectively. The hypomethylation threshold (defined as the 99th percentile of normal thyroid tissues) is indicated by a horizontal dashed line. *$P < 0.001$ relative to normal thyroid tissues. M1 DTC indicates distant metastatic DTC.

PUMA was similar among *BRAF* and *RAS* mutated tumors, as well as compared with tumors WT for both *BRAF* and *RAS*.

PUMA and clinical data

Within normal thyroid tissues, PUMA did not correlate with age ($P = 0.762$). Conversely, in tumor tissues PUMA was correlated with age ($P < 0.001$), but after separate analyses in strata of thyroid cancer risk category (*i.e.*, low-risk DTC, pediatric PTC, distant metastatic DTC, and PDTC/ATC), this association was lost, indicating that PUMA is associated with thyroid cancer risk category rather than age.

Kaplan–Meier analyses showed that the endpoints thyroid cancer–related and all-cause mortality were affected by the hypomethylation state of the primary tumor (log rank $P < 0.001$ for both, Fig. 2). In unadjusted Cox regression analyses, each percentage increase in PUMA was associated with a hazard ratio (HR) of 1.11 [95% confidence interval (CI), 1.06 to 1.15] for both mortality endpoints. This association remained significant after adjustment for age, but was lost after further adjustment for thyroid cancer risk category: HR (95% CI), 1.02 (0.95 to 1.09) and 1.02 (0.96 to 1.10) for thyroid cancer–related and all-cause mortality, respectively (Table 4). Interestingly, although the series had limited statistical power, Kaplan–Meier curves for patients with distant metastatic DTC (Supplemental Fig. 6) suggested that survival was different according to the hypomethylation status in the first 6 years of follow-up. However, after this time, the survival curves crossed each other. Finally, in Supplemental Fig. 7, Kaplan–Meier curves for non-PDTC/ATC patients by quartiles of PUMA showed that patients in the highest quartiles had the worst survival.

Table 2. PUMA and Tumor Hypomethylation Status Represented for Normal Thyroid Tissue and by Thyroid Cancer Risk Category

	Normal Thyroid Tissue	Low-Risk DTC	M1 DTC	PDTC/ATC	Pediatric PTC
N	20	28	33	16	13
PUMA, median (IQR)	2.8 (2.3–3.2)	2.9 (2.6–3.5)	4.0 (3.1–6.2)	9.3 (7.0–12.1)	3.2 (2.9–3.6)
Hypomethylated tumors, n (%)	—	1 (3.6)	14 (42.4)	15 (93.8)	1 (7.7)

M1 DTC indicates distant metastatic DTC.

Table 3. Identified Mutations, Represented by Tumor Histology and Tumor Risk Classification (Pediatric Tumors Excluded), and Median [IQR] PUMA Presented by Mutation Status

		PTC		FTC			
Mutation Status	Total (N = 77)	Low-Risk (n = 20)	Distant Metastatic (n = 20)	Low-Risk (n = 8)	Distant Metastatic (n = 13)	PDTC/ATC (n = 16)	Median PUMA (IQR)
BRAF mutated[a]	14	9	4	0	0	1	3.3 (2.6–5.1)
RAS mutated[b]	11	1	2	1	4	3	3.5 (2.6–7.0)
BRAF and RAS WT	40	8	7	7	7	11	4.1 (2.9–8.2)
Unknown	12	2	7	0	2	1	3.7 (3.0–6.8)

[a] All BRAF p.Val600Glu mutations.

[b] Eight tumors with NRAS c.182A>G (p.Gln61Arg), one with NRAS c.181C>A (p.Gln61Lys), one with HRAS c.37G>C (p.Gly13Arg), and one with HRAS c.182A>G (p.Gln61Arg) mutations.

Discussion

One of the hallmarks of cancer is global DNA hypomethylation. In the present study we assessed global Alu hypomethylation as a surrogate marker for global DNA hypomethylation in a broad prognostic spectrum of thyroid cancers, and we found that Alu hypomethylation was increasingly affected in distant metastatic DTC, PDTC, and ATC. Conversely, hypomethylation was not increased in low-risk DTC and pediatric PTC, and no further hypomethylation was observed in distant metastases as compared with the matched primary tumors.

DNA global hypomethylation in thyroid cancer has been assessed in several studies (25, 28–32), but these showed conflicting results with regard to the presence of global DNA hypomethylation. This can be explained by the low number of tumor samples that have been included in these studies, the poor characterization of thyroid tumors, and the usage of different techniques for assessment of global DNA hypomethylation. In the present study, we analyzed a large series of thyroid tumors in a broad prognostic range. To the best of our knowledge, this is the first study in which global Alu hypomethylation is assessed in thyroid tumors of pediatric DTC patients, as well as patients with distant metastatic DTC, PDTC, and ATC. We used the recently developed QUAlu technique, which quantifies global Alu hypomethylation, and showed that it is easily applicable to a wide range of DNA quantities derived from FFPE tissues, including very low amounts of partly degraded DNA that is often encountered in pathological examination specimens. Furthermore, we confirmed that results were comparable to those from fresh-frozen tissues.

Our findings revealed that global hypomethylation of Alu repeats seems to be a late event in thyroid cancer, as it occurred in 42% of distant metastatic DTCs, and in most PDTCs and ATCs, whereas it was virtually absent in low-risk DTCs. On the contrary, Timp *et al.* (32) showed that the hypomethylation of large genome blocks was a very early event, being present in benign thyroid lesions and follicular adenomas. This apparent discordance may be explained by the use of a different methodology that covered unique sequences but not repetitive elements. Within DTCs, PTCs and FTCs had a similar behavior with regard to global Alu hypomethylation. In a prior study we found that FTC had higher levels of methylation than did PTC (9), but only promoter regions were analyzed. Alternatively, we did not find an increased Alu hypomethylation in distant metastases as compared with the matched primary tumor, which implies that tumor hypomethylation remains relatively stable during distant metastatic spread in thyroid carcinoma patients.

The increasing Alu hypomethylation in distant metastatic DTC, PDTC, and ATC suggests that global DNA hypomethylation might be implicated in tumor progression or cell dedifferentiation. This is further illustrated by the PUMA values of the cell lines analyzed. These values were best fit in the range of PDTC and ATC, which corresponds to the finding that thyroid cancer cell lines (either derived from PTC, FTC, or ATC) show characteristics of dedifferentiated cells (with a gene expression resembling that of undifferentiated tumors, absence of thyroid-specific gene expression, and loss of thyrotropin sensitivity) (33). However, it remains unknown whether global DNA hypomethylation acts as an oncogenic driver or is a consequence of the overall genome deregulation in cancer. The prior was suggested in a mouse model in which induction of hypomethylation led to chromosomal instability and tumor development (34). Furthermore, a direct link between DNA hypomethylation and genomic instability as well as poor survival has been reported in human colorectal cancer (35, 36).

DTC is epigenetically distinct based on *BRAF* and *RAS* mutations (9–11). Accordingly, even though we did not find any relationship with PUMA when analyzing all tumors, we found that *BRAF* mutated distant metastatic DTCs were hypomethylated, but not *RAS* mutated tumors. Interestingly, tumors WT for both *BRAF* and *RAS*

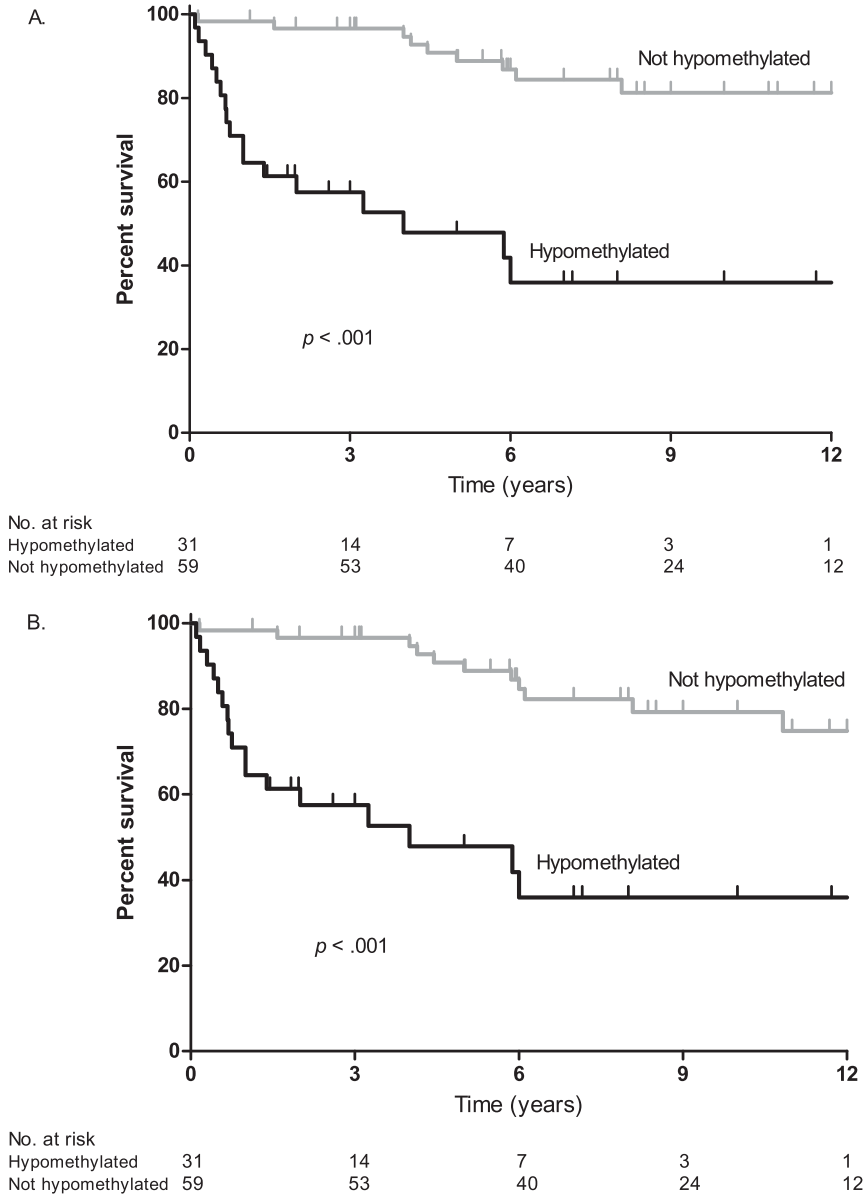

Figure 2. Kaplan–Meier survival curves of (A) thyroid cancer–related survival and (B) all-cause mortality, by methylation status of the primary tumor (black line indicates hypomethylated; gray line indicates not hypomethylated).

showed a wide range of PUMAs, which might reflect the *BRAF*- and *RAS*-like phenotypes described by The Cancer Genome Atlas consortium (11). Additional studies are required to confirm it. Owing to low statistical power, further analyses within thyroid cancer risk categories were limited. Furthermore, data on other mutation then BRAF and RAS were not available. Still, these results suggest distinct molecular mechanisms underlying the tumorigenesis of *BRAF* and *RAS* mutated distant metastatic DTCs.

Several studies from the last three decades have clearly demonstrated that aging is associated with a genome-wide decrease of DNA methylation (37). To exclude that the association we found between thyroid cancer and global hypomethylation was an age-related event, we assessed the correlation between age and PUMA within thyroid cancer risk category strata. In this study, no significant correlations were found, suggesting that global Alu hypomethylation was dependent on risk classification rather than on age. Pediatric DTC showed a similar hypomethylation as adult normal thyroid tissues, which could reflect the well-differentiated character of these tumors (2). Therefore, Alu hypomethylation does not seem to play an important role in the relative aggressiveness of pediatric PTC. However, we cannot rule out that lower cutoff levels for hypomethylation should be applied for pediatric patients.

Finally, we found that Alu hypomethylation of primary thyroid tumors was associated with both thyroid cancer–related and all-cause mortality in unadjusted and age-adjusted Cox regression analyses. However, the association was lost after adjustment for thyroid cancer risk category, which precludes the use of

Table 4. Unadjusted and Adjusted Cox Regression Models for the Endpoints Thyroid Cancer–Related and All-Cause Mortality by PUMA

	Thyroid Cancer–Related Mortality		All-Cause Mortality	
Cox Regression Model	**HR (95% CI)**	***P* Value**	**HR (95% CI)**	***P* Value**
Unadjusted PUMA, per 1% increase	1.11 (1.06–1.15)	<0.001	1.11 (1.06–1.15)	<0.001
Age-adjusted PUMA, per 1% increase	1.07 (1.01–1.13)	0.014	1.07 (1.01–1.13)	0.015
Age- and risk category-adjusted PUMA,[a] per 1% increase	1.02 (0.95–1.09)	0.637	1.02 (0.96–1.10)	0.505

[a]Adjusted for age and thyroid cancer risk category.

PUMA as an independent biomarker for these endpoints in clinical practice. This is in accordance with the overlap of PUMA in thyroid cancer risk categories. Interestingly, PUMA of DTC that developed distant metastases during follow-up (*i.e.*, not present at diagnosis) was in the same range as that of tumors with distant metastatic spread at diagnosis. In future research it would therefore be interesting to study whether PUMA has a predictive value for development of distant metastases.

In addition to Alu repeats, global DNA hypomethylation is commonly assessed in LINE-1 as a marker in cancer (38). Although both LINE-1 and Alu repeats are highly repetitive sequences that comprise a substantial part of the genomic mass, hypomethylation of these elements is not necessarily the same in a tumor. There may be a cancer-specific hypomethylation profile of repetitive elements (16, 39). We chose to study Alu repeats because these elements contain 25% of all CpG sites, are mostly located in gene-rich regions, and are relatively short elements that can be easily assessed using the QUAlu technique, even in FFPE samples that contain partly degraded DNA. Nonetheless, in further studies it would be interesting to additionally evaluate LINE-1 hypomethylation in different stages of thyroid cancer.

Global DNA hypomethylation coexists with focal hypermethylations and hypomethylations. In thyroid cancer, as in most cancers, a large number of targeted changes in DNA methylation have been identified and proposed as biomarkers (40). Further analyses aiming to design a panel that incorporates global and specific DNA methylation alterations could improve the clinical value of these potential biomarkers.

In conclusion, we found an increasing Alu hypomethylation in distant metastatic DTC, PDTC, and ATC, whereas low-risk DTC and pediatric PTC were not affected by hypomethylation. This might reflect the involvement of global hypomethylation in a subset of thyroid cancers and its association with advanced disease and cell dedifferentiation. Alu hypomethylation seems to be rather stable during distant metastatic spread. Further studies are warranted to clarify its contribution to malignant behavior, the interplay with other genetic and epigenetic alterations, as well as its potential application in preoperative fine needle aspiration cytology as a diagnostic and/or prognostic marker.

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