REVIEW

DNA methylation in thyroid cancer

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Abstract

In recent years, cancer genomics has provided new insights into genetic alterations and signaling pathways involved in thyroid cancer. However, the picture of the molecular landscape is not yet complete. DNA methylation, the most widely studied epigenetic mechanism, is altered in thyroid cancer. Recent technological advances have allowed the identification of novel differentially methylated regions, methylation signatures and potential biomarkers. However, despite recent progress in cataloging methylation alterations in thyroid cancer, many questions remain unanswered. The aim of this review is to comprehensively examine the current knowledge on DNA methylation in thyroid cancer and discuss its potential clinical applications. After providing a general overview of DNA methylation and its dysregulation in cancer, we carefully describe the aberrant methylation changes in thyroid cancer and relate them to methylation patterns, global hypomethylation and gene-specific alterations. We hope this review helps to accelerate the use of the diagnostic, prognostic and therapeutic potential of DNA methylation for the benefit of thyroid cancer patients.

Key Words

- thyroid cancer
- DNA methylation
- global hypomethylation
- biomarkers
- demethylating drugs

Introduction

Thyroid cancer, the most prevalent endocrine malignancy, covers the full range of phenotypes from indolent to the worst forms of human cancer. It is categorized into differentiated thyroid cancer (DTC), poorly differentiated thyroid cancer (PDTC) and undifferentiated or anaplastic thyroid cancer (ATC), all of which are derived from thyroid follicular cells, and into medullary thyroid cancer (MTC), which is derived from parafollicular cells. Moreover, DTC has three basic subtypes: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC) and Hurthle cell thyroid cancer (HCTC). Globally, DTC accounts for 95% of all thyroid carcinomas. During the last few decades, several epidemiologic studies have reported that DTC incidence has increased worldwide (Lim et al. 2017). The reasons for this are not clear; it has been attributed both to a true increase in the incidence of DTC and to the improvement and more extensive use of imaging techniques such as neck ultrasound (Kitahara et al. 2017).

Currently, total thyroidectomy, the removal of the affected neck lymph nodes of the central compartment, radioiodine (RAI) therapy for the ablation of thyroid remnants or metastases and TSH suppression with l-thyroxin are the treatment schedule for a large portion of DTC patients (Haugen et al. 2016). With these therapeutic approaches, the majority of DTC patients exhibit good prognosis with a >98% 5-year survival rate. However, a subset of tumors progress to display more aggressive behavior, and some of these undergo a progressive process of dedifferentiation that makes them less capable of producing thyroglobulin and concentrating iodine, producing a poor response to RAI. To identify patients with a progressive course of the disease, a number of
prognostic factors and clinical scores have been proposed that are mainly age, the histological variant, the initial extent of the disease and the size of the primary tumor (Asa 2017). However, these prognostic factors have some limitations.

In recent years, the management of thyroid cancer in patients is shifting toward more personalized medicine to avoid the overdiagnosis and overtreatment of tumors with an indolent course and, at the same time, to identify those tumors that will progress (Dralle et al. 2015). The final goal is to deliver the most effective but least aggressive treatment. A better understanding of the molecular mechanisms underlying thyroid cancer progression may be key to tailor the management of this disease. In this regard, significant progress has been made in the last 20 years (Riesco-Eizaguirre & Santisteban 2016). The major event in PTC carcinogenesis is the constitutive activation of mitogen-activated protein kinase (MAPK), whereas the PI3K/AKT pathway is involved in the progression of FTC. Recently, the genetic landscape of some thyroid cancer histotypes has been largely deciphered (Cancer Genome Atlas Research Network 2014, Kunstman et al. 2015), and some of these genetic alterations have been used both as diagnostic tools (in the study of thyroid nodules) and as prognostic tools. Importantly, for the first time, the last set of American Thyroid Association (ATA) guidelines recommended the use of mutations in the BRAF gene and the TERT promoter as prognostic factors in PTC (Haugen et al. 2016).

However, cancer is not only caused by genetic abnormalities but also by epigenetic alterations (reviewed in Jones & Baylin 2007). The most widely used definition for epigenetics is ‘the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence’ (Wu & Morris 2001, Bird 2007). Epigenetics can explain how two identical genotypes can lead to different phenotypes. There are several epigenetic mechanisms: DNA methylation, posttranslational modifications of histones, chromatin remodeling or non-coding RNAs. These mechanisms have been reviewed elsewhere (Bannister & Kouzarides 2011, Holoch & Moazed 2015, Lägst & Manelyte 2015, Allis & Jenuwein 2016, Feinberg et al. 2016), and here we will focus on DNA methylation.

**What is DNA methylation?**

DNA methylation was the first discovered epigenetic modification (Hotchkiss 1948, Holliday & Pugh 1975, Riggs 1975) and consists of the covalent addition of a methyl group to the 5-carbon of the cytosine, giving rise to 5-methylcytosine (5mC) (reviewed in Portela & Esteller 2010). In humans, DNA methylation occurs almost exclusively within CpG dinucleotides, which are underrepresented (i.e., found in a lower than expected proportion based on the G/C content) and not evenly distributed throughout the genome (Bird 1980). Most human genome (approximately 60–80% of CpG sites) is methylated, except for some CpG-rich regions called CpG islands (CGIs), which are often unmethylated and encompass the promoters of approximately 60% of protein-coding genes (Ehrlich et al. 1982, Bird 1986, Lister et al. 2009).

DNA methylation is frequently described as a repressive epigenetic mark. However, DNA methylation function varies depending on the genomic context (reviewed in Jones 2012, Baubec & Schubeler 2014) (Supplementary Fig. 1, see section on supplementary data given at the end of this article). DNA methylation in proximal and distal regulatory elements (i.e., promoters and enhancers, respectively) represses transcription by affecting the binding of transcription factors and/or recruiting enzymes that modify chromatin structure. Conversely, DNA methylation of the gene body may enhance transcriptional elongation and affect splicing. In the case of repetitive elements, which are densely methylated, DNA methylation is the major repression mechanism.

Therefore, DNA methylation is a key player in the regulation of gene expression and is implicated in many cellular processes such as imprinting (Reik et al. 1987, Swain et al. 1987), X-chromosome inactivation (Mohanadas et al. 1981) and the establishment and maintenance of cell type-specific expression programs (reviewed in Suvels et al. 2016). DNA methylation is also essential for the maintenance of genome stability by modeling chromatin structure (reviewed in Madakashira & Sadler 2017) as well as by silencing repetitive sequences to prevent chromosomal rearrangements (Gaudet et al. 2003) and the expression and expansion of transposable elements (reviewed in Belancio et al. 2010).

Furthermore, it is noteworthy that DNA methylation is an important source of promising cancer biomarkers for many reasons: DNA methylation is stable even in fixed samples over time, easily detected by well-established techniques (Fig. 1 and Supplementary Table 1), present in various bodily fluids, and cell type specific (Koch et al. 2018).
Writers, erasers and readers of DNA methylation

DNA methylation does not function alone but is involved in a complex crosstalk with many other players to reinforce specific regulatory programs. Although how DNA methylation is interpreted in the context of genome regulation is not completely understood, there are some proteins known to modulate DNA methylation. They are classified as writers, erasers and readers of DNA methylation.

DNA methylation writers are proteins that establish and maintain DNA methylation patterns through development and differentiation. These proteins, called DNA methyltransferases (DNMTs), transfer a methyl group to cytosine residues (reviewed in Goll & Bestor 2005) (Fig. 2).

Despite the high stability of DNA methylation, 5mC can be demethylated by passive or active mechanisms, the latter mediated by erasers that generate DNA demethylation intermediates, such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (reviewed in Wu & Zhang 2017) (Fig. 2). Interestingly, although the levels of 5hmC in human cells are very low (in general, 14-fold lower than the levels of 5mC) and vary greatly between different tissues, 5hmC is present at a relatively stable abundance, suggesting that it is not just...
a DNA demethylation intermediate. 5hmC is associated with gene transcription, although this relationship is not fully understood (reviewed in Shi et al. 2017) (Supplementary Fig. 1).

Finally, DNA methylation readers are proteins that specifically bind to methylated CpGs and coordinate the crosstalk between DNA methylation, histone modifications and chromatin organization to reinforce downstream regulatory programs. The paradigm of these proteins are the methyl-CpG-binding domain (MBD) family proteins, which have the ability to recruit chromatin remodelers, histone deacetylases (HDAC) and DNMTs to methylated CpGs associated with gene repression (reviewed in Du et al. 2015) (Supplementary Fig. 1).

**Aberrant DNA methylation is a hallmark of cancer**

Disruption of DNA methylation is a common feature in human disease, both in noncancerous diseases and in cancer (Fernandez et al. 2012). Although the first discovered alteration of DNA methylation in cancer was an overall reduction in 5mC levels, that is, a global hypomethylation in tumoral cells compared to the methylation in normal cells (Feinberg & Vogelstein 1983, Gama-Sosa et al. 1983), this epigenetic alteration has been ignored for decades mainly due to the technical complexity of its analysis. Conversely, research has focused on hypermethylation (i.e., the increase in the methylation of CpG sites compared to normal cells), which often but not exclusively occurs in localized sequences associated with CGIs (Herman et al. 1995, Melki et al. 1999, Esteller et al. 2000, Nguyen et al. 2001). Both global hypomethylation and focal hypermethylations are constant features of the cancer genome and often coexist in tumoral cells, but although there is interplay between them, their underlying mechanisms seem to be independent.

The main consequence of the hypermethylation of promoters and enhancers is the repression of the expression of genes functionally important in the neoplastic process, whose silencing may have a tumor-promoting effect. The hypermethylation profile is tumor specific and affects all cellular pathways (reviewed in Esteller 2007). Some genes such as p16INK4A and MLH1 are frequently hypermethylated in many cancers including thyroid cancer (Schagdarsurengin et al. 2006, Guan et al. 2008) while others are tumor specific (e.g., the sodium iodide symporter gene – SLC5A5 or NIS – in thyroid cancer) (Neumann et al. 2004, Galrão et al. 2014). In contrast to global hypomethylation, an overall increase in
in 5mC levels in tumors compared to its levels in normal tissues is much less common, despite the high number of local hypermethylations (Ehrlich 2002).

For several decades, it has been widely accepted that the hypomethylation of repetitive sequences is responsible for global hypomethylation (Ehrlich 2009). However, recent approaches enabling the mapping of DNA methylation at the genome scale have shown that global hypomethylation affects large genome domains including both repetitive and unique sequences (Berman et al. 2011, Hansen et al. 2011, Timp et al. 2014). In this context, hypomethylation can encompass regulatory elements and affect gene expression (reviewed in Wilson et al. 2007). Nevertheless, hypomethylation-dependent transcriprional activation is less frequent than hypermethylation-dependent transcriptional silencing. In contrast, numerous studies indicate that global hypomethylation is associated with chromosomal instability and the reactivation of transposable elements (Gaudet et al. 2003).

5hmC is also perturbed in cancer in a similar way as 5mC, i.e., there is a strong global loss of this epigenetic mark in tumors. However, its involvement in thyroid cancer is completely unknown. A recent study that analyzed 5hmC in circulating cell-free DNA and in tumoral and normal tissues from different cancer types, including thyroid cancer, found that 5hmC was mainly distributed in transcriptionally active regions (Li et al. 2017). Importantly, they identified cancer-specific 5hmC signatures. To the best of our knowledge, this is the only study of 5hmC in thyroid cancer; thus, 5hmC has opened a new field to explore in this disease.

Disruption of epigenetic pathways in cancer

The recent whole exome sequencing of thousands of tumoral samples of different cancer types has revealed that many genes controlling the epigenome are mutated, which can lead to epigenetic aberrations (reviewed in You & Jones 2012). This is the case for mutations in the writers, erasers and readers of DNA methylation. Mutations in the DNMT and TET genes have been identified in different cancers; for example, DNMT3A and TET2 are frequently mutated in hematologic malignancies. In thyroid cancer, mutations in these genes are rare (<1.5% in PTC and <3% in ATC and PDTC) (data from The Cancer Genome Atlas Research Network; http://www.cbioportal.org/) (Cerami et al. 2012). However, the expression of some of these genes is altered (Supplementary Fig. 2) and could contribute to the dysregulation of DNA methylation in thyroid cancer, although further studies should be performed to understand the underlying relationship. On the other hand, MBD proteins are mutated in several cancers, including PTC (Du et al. 2015). Although they represent less than 5% of patients, the study of these proteins in thyroid cancer could be a promising field.

DNA methylation changes in thyroid cancer: drivers of disease progression and biomarkers

DNA methylation has been extensively studied in many cancers, such as colorectal and breast cancer, and due to technological advances, enormous progress has been made in the understanding of the epigenetic landscape of these tumors. Conversely, the role of DNA methylation in thyroid cancer has received comparatively less attention (Fig. 3). The first DNA methylation studies in thyroid cancer were based on candidate gene approaches assessing the DNA methylation levels of specific gene promoters (Supplementary Table 2). It was not until 2011 that Hou et al. performed the first array-based, genome-wide DNA methylation study using two PTC cell lines to analyze the effect of the BRAF(V600E) mutation on DNA methylation (Hou et al. 2011). To our knowledge, 11 more array-based studies using different platforms (Goldengate, 27K or 450K) to profile the methylomes of tissue samples from patients with thyroid cancer have been published since then (Table 1). All these studies showed that thyroid cancer is not an exception and exhibits DNA methylation alterations. However, different pan-cancer analyses based on data from the Cancer Genome Atlas Research Network revealed that PTC has one of the lowest frequency of DNA methylation alterations. Specifically, Yang et al. performed a differential DNA methylation analysis between normal and tumoral samples (n=5480) for 15 cancer types showing high variability in the numbers of differentially methylated CpGs, which ranged from 3722 in PTC to 57,290 in uterine corpus endometrial carcinoma (Yang et al. 2016a). Accordingly, another DNA methylation pan-cancer study focused on promoters found that PTC exhibited one of the lowest frequencies in both hypomethylation and hypermethylation events (Saghaﬁnia et al. 2018) (Fig. 4). In addition, these authors introduced the concept of DNA methylation instability, which was found to be very low in PTC. In contrast, ATC exhibits a high frequency of DNA methylation alterations (10-fold higher than PTC; Bisarro dos Reis et al. 2017).
Interestingly, these epigenetic differences between PTC and ATC also can be found at the level of genetic alterations. A recent pan-cancer analysis on whole exome sequencing revealed that the mutation frequency in PTC was one of the lowest (approximately 1 change/Mb across the entire exome) among solid tumors (Lawrence et al. 2013) (Fig. 4), while the mutation frequency in ATC was at the opposite extreme and was closer to that in melanoma and lung cancer, exceeding 100 changes/Mb (Kunstman et al. 2015, Riesco-Eizaguirre & Santisteban 2016). As mutations are largely caused by errors in DNA replication (Tomasetti et al. 2017), some researchers propose that the cell division rate also participates in shaping the cancer DNA methylation landscape (Yang et al. 2016b). Thus, the different proliferation rate between PTC (low) and ATC (high) could explain, at least in part, the different frequencies of their DNA methylation alterations.

**Thyroid cancer DNA methylation patterns**

Since the initial studies assessing the DNA methylation of a single locus, there has been accelerating technological progress providing a plethora of DNA methylation techniques that have allowed the generation of single CpG resolution maps (Fig. 1 and Supplementary Table 1). These maps have improved our understanding of DNA methylation and have shown that DNA methylation patterns, the so-called methylomes, are tissue specific, allowing us to distinguish different normal tissues from each other (Hansen et al. 2011, Fernandez et al. 2012). Moreover, methylomes differ largely between normal and tumoral cells and between different types of tumors, which is key from a translational point of view. An example of the clinical use of this specificity is that methylomes allow the identification of the tissue of origin in carcinomas of unknown primary origin (CUPs) (Moran et al. 2016).

**Association between methylomes and histology in thyroid cancer**

Genome-wide studies to profile thyroid cancer methylomes, most of which used BeadArrays (Table 1), revealed histology-associated DNA methylation profiles. Specifically, PTC is characterized by a higher number of hypomethylations (most of them outside promoter regions) than hypermethylations in comparison to normal thyroid tissues (Ellis et al. 2014, Mancikova et al. 2014, White et al. 2016, Beltrami et al. 2017, Bisarro dos Reis et al. 2017) (Supplementary Fig. 3). Only the study by Rodríguez-Rodero et al. identified more hypermethylation than hypomethylation, which could be explained by the low number of analyzed PTCs (Rodriguez-Rodero et al. 2013). In contrast to PTC, FTC exhibits more hypermethylation than hypomethylation (most of them outside promoter regions) (Rodriguez-Rodero et al. 2013, Mancikova et al. 2014, Bisarro dos Reis et al. 2017, Affinito et al. 2019) as well as follicular adenomas (FA), although the number of DNA alterations in these benign tumors is low, thus resembling normal thyroid methylomes (Supplementary Fig. 3). There is debate within the field about whether FA and FTC are distinct molecular entities or represent a biological continuum (Arora et al. 2008, Krause et al. 2011, Yoo et al. 2016). Interestingly, Mancikova et al. showed that most of the FA-associated promoter hypermethylation that they identified were also found in FTC, suggesting a progressive gain of hypermethylation along the tumorigenic process from adenomas to carcinomas, thereby reinforcing the hypothesis that some FAs have the malignant potential
Table 1  Summary of studies in thyroid cancer using methods for genome-wide analysis of DNA methylation.

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Method</th>
<th>Discovery series (n)</th>
<th>Integration expression</th>
<th>Identified genes of interest</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCA/CGI array GoldenGate</td>
<td>Cell lines (2)</td>
<td>NA</td>
<td>NA</td>
<td>RT-qPCR, GEO array data</td>
</tr>
<tr>
<td>2</td>
<td>24130 27K</td>
<td>NT (25); TC (36)</td>
<td>NA</td>
<td>NA</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>3</td>
<td>174</td>
<td>NT (10); PTC (14)</td>
<td>309</td>
<td>14</td>
<td>GEO array data</td>
</tr>
<tr>
<td>4</td>
<td>450K</td>
<td>NT (8); FA (18)</td>
<td>89</td>
<td>9</td>
<td>GEO array data</td>
</tr>
<tr>
<td>5</td>
<td>NT (2)</td>
<td>PTC (2); FTC (2); ATC (2) cell lines (4)</td>
<td>408</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>1531</td>
<td>NT (8); FTC (29); vPTC (15) recurrent PTC (7); tcPTC (35); Other PTC (38)</td>
<td>255</td>
<td>2582</td>
<td>GEO array data</td>
</tr>
<tr>
<td>7</td>
<td>27K</td>
<td>NT (56); CPTC (324); vPTC (99); tcPTC (35); Other PTC (38)</td>
<td>164</td>
<td>405</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>83</td>
<td>NT (8); FA (18); FTC (38)</td>
<td>255</td>
<td>2582</td>
<td>GEO array data</td>
</tr>
<tr>
<td>9</td>
<td>408</td>
<td>NT (16); PTC (13)</td>
<td>408</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
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<td>NT (41); PTC (41)</td>
<td>645</td>
<td>5425</td>
<td>GEO array data</td>
</tr>
<tr>
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<td>NT (50); FTC (60); PTC (60); FTC (1); PTC (1); ATC (3)</td>
<td>400</td>
<td>1475</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>28252</td>
<td>NT (7); FA (10); FTC (11)</td>
<td>28252</td>
<td>–</td>
<td>GEO array data</td>
</tr>
<tr>
<td>13</td>
<td>907</td>
<td>NT (3); PTC (3); BTLs (28)</td>
<td>907</td>
<td>–</td>
<td>GEO array data</td>
</tr>
<tr>
<td>14</td>
<td>907</td>
<td>NT (39); BTLs (28); PTC (39)</td>
<td>907</td>
<td>–</td>
<td>GEO array data</td>
</tr>
</tbody>
</table>

*Hyper, number of hypermethylation events compared to normal tissue; Hypo, number of hypomethylation events compared to normal tissue.

2K, Infinium HumanMethylation27 BeadChip; 450K, Infinium HumanMethylation450 BeadChip; ATC, anaplastic TC; BTLs, benign thyroid lesions; cPTC, classical PTC; DTC, differentiated TC; FA, follicular adenoma; FTC, follicular TC; HSCT, Hurthle cell TC; NT, normal tissue; PDTC, poorly differentiated TC; PTC, papillary TC; RRBS, reduced representation bisulfite sequencing; TC, thyroid cancer; tcPTC, tall cell PTC.

To give rise to FTC (Mancikova et al. 2014). Accordingly, unsupervised clustering analysis in the study by Bisarro dos Reis et al. showed that FAs clustered with FTCs, and a recent study by Affinito et al. found that FAs displayed an intermediate DNA methylation profile between FTCs and normal thyroid tissues (Bisarro dos Reis et al. 2017, Affinito et al. 2019).

The majority of genome-wide studies are focused on PTC. Interestingly, some of them specify the PTC variants used, revealing differential DNA methylation profiles.
Ellis et al. found that classical PTC (cPTC) displayed a high number of DNA alterations, most of which were hypomethylations, whereas follicular variant of PTC (fvPTC) exhibited a smaller proportion of hypomethylations (Ellis et al. 2014). In this regard, this study, as well as those by Mancikova et al. and the Cancer Genome Atlas Research Network, found that fvPTC exhibited a methylome that was not as different from that of normal thyroid tissue (Mancikova et al. 2014, Cancer Genome Atlas Research Network 2014). 

Apart from cPTC and fvPTC, the Cancer Genome Atlas Research Network also analyzed tall cell PTC (tcPTC). Based on an unsupervised clustering analysis, they classified tumors into four groups: two groups enriched by fvPTC (Meth-follicular, which exhibited few methylation changes compared to normal tissue and Meth-CGI, which was characterized by the hypermethylation of numerous CGIs) and two groups enriched by cPTC and tcPTC (Meth-classical 1 and Meth-classical 2, which were characterized by hypomethylation outside of CGIs). Interestingly, a small subset of fvPTCs resembled tcPTC and cPTC. Mancikova et al., who also included FA and FTC in the study, showed that fvPTC methylomes were more similar to follicular tumors than to cPTC (Mancikova et al. 2014). These results indicate that PTCs with follicular architecture are different from PTCs with papillary architecture. In the future, it will be of great interest to investigate whether the new noninvasive follicular neoplasm with papillary-like nuclear features (NIFTP) entity shows a specific methylation profile.

Two of the array-based studies included several PDTC cancers. The x-axis gives the number of samples compared to normal tissues. (C) Somatic mutation frequencies in 6010 human tumors across 27 cancer types. Tumor types are sorted by their median hypermethylation, hypomethylation and somatic mutation frequencies. Papillary thyroid tumors (red bars) are among tumors with the lowest frequency of epigenetic and genetic alterations, mostly leukemias and pediatric cancers. The x-axis gives the number of samples for each tumor type. Data from Lawrence et al. (2013) and Saghafinia et al. (2018). ACC, adrenocortical carcinoma; AML, acute myeloid leukemia; BLCA, bladder carcinoma; BRCA:B, basal breast invasive carcinoma; BRCA:L, luminal breast invasive carcinoma; Car, carcinoid tumors; CESC, cervix squamous cell carcinoma; CLL, chronic lymphocytic leukemia; CRC, colon and rectum carcinoma; DLBCL, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; EwingSRCC, Ewing sarcoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe carcinoma; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LGG, low grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MD, medulloblastoma; NB, neuroblastoma; MM, multiple myeloma; OV, ovarian carcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; PTC, papillary thyroid cancer; RhD, Rhabdoid tumor; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; UCS, uterine corpus endometrial carcinoma; UCEC, uterine cervix carcinoma.

**Figure 4**

Epigenetic and genetic alterations by tumor type. (A) Hypermethylation and (B) hypomethylation event frequencies in 6101 human tumors across 24 cancer types. Frequencies are estimated as the percentage of probes found hypermethylated (out of 64,414) or hypomethylated (out of 3423) compared to normal tissues. (C) Somatic mutation frequencies in 3025 tumor-normal sample pairs across 27 cancer types. Tumor types are sorted by their median hypermethylation, hypomethylation and somatic mutation frequencies. Papillary thyroid tumors (red bars) are among tumors with the lowest frequency of epigenetic and genetic alterations, mostly leukemias and pediatric cancers. The x-axis gives the number of samples for each tumor type. Data from Lawrence et al. (2013) and Saghafinia et al. (2018). ACC, adrenocortical carcinoma; AML, acute myeloid leukemia; BLCA, bladder carcinoma; BRCA:B, basal breast invasive carcinoma; BRCA:L, luminal breast invasive carcinoma; Car, carcinoid tumors; CESC, cervix squamous cell carcinoma; CLL, chronic lymphocytic leukemia; CRC, colon and rectum carcinoma; DLBCL, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; EwingSRCC, Ewing sarcoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe carcinoma; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LGG, low grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MD, medulloblastoma; NB, neuroblastoma; MM, multiple myeloma; OV, ovarian carcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; PTC, papillary thyroid cancer; RhD, Rhabdoid tumor; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; UCS, uterine corpus endometrial carcinoma; UCEC, uterine cervix carcinoma.
While the study by Rodríguez-Rodero et al. identified fewer DNA methylation alterations in ATC than in DTC, Bisarro dos Reis et al. identified a drastically higher number of DNA methylation alterations in PDTC and ATC (six-fold higher than in FTC and ten-fold higher than in PTC) (Rodríguez-Rodero et al. 2013, Bisarro dos Reis et al. 2017). The contradictory results of these two studies are probably due to the low number of analyzed samples and the use of different arrays (27K vs 450K platforms) that cover different regions of the genome (Rodríguez-Rodero et al. 2013, Bisarro dos Reis et al. 2017). However, both studies concluded that PDTC and ATC exhibited more hypomethylation than hypermethylation events, suggesting the association of hypomethylation with dedifferentiation. The similarity between the methylenomes of ATC, PDTC, extensively invasive FTC and lymphocytic thyroiditis found by Bisarro dos Reis et al. is noteworthy (Bisarro dos Reis et al. 2017); as these aggressive tumors are characterized by a high level of immune cell infiltration (Ryder et al. 2008), these results suggest that part of the DNA methylation alterations in these tumors may come from infiltrating immune cells.

BeadArrays are widely used in genome-wide DNA methylation studies due to their low cost, but they are limited to the CpGs covered by the array. However, reduced representation bisulfite sequencing (RRBS), which is based on next-generation sequencing, has a higher sensitivity, resolution and coverage than BeadArrays. There are two studies that investigated PTC methylomes using RRBS (Table 1). Zhang et al. focused on the methylation of mRNA and lncRNA promoters and confirmed previous results showing more hypomethylation than hypermethylation events in PTC (Zhang et al. 2017). However, when DNA methylation and expression data from the same samples were crossed, only 19 mRNAs were upregulated/hypomethylated, and 26 mRNAs and 3 lncRNAs were downregulated/hypermethylated. These findings, which are consistent with results from Mancikova et al. and Affinito et al. (Mancikova et al. 2014, Affinito et al. 2019), suggested that DNA methylation in promoters does not have a widespread role in controlling gene expression in PTC. On the other hand, by using RRBS, Yim et al. identified a unique DNA methylation signature of 4,575 CpGs specific to benign nodules, most of which were hypermethylated compared to adjacent normal tissues and malignant nodules, that may have important diagnostic applications (Yim et al. 2019). They also analyzed specimens with lymphocytic thyroiditis and, in accordance with the results from Bisarro dos Reis et al., suggested that the presence of immune-infiltrating cells in tumors may affect DNA methylation patterns (Bisarro dos Reis et al. 2017).

Globally, most DNA methylation alterations in thyroid cancer occur outside promoter regions and are specifically associated with histology.

**Association between DNA methylation and genetic drivers in thyroid cancer**

Another important finding derived from these studies is the relationship between DNA methylation profiles and mutations in BRAF and RAS genes; BRAF-mutated tumors harbor more hypomethylations (which is expected since this mutation is almost exclusively detected in PTC), while RAS-mutated tumors harbor more hypermethyllations (which is expected since this mutation mostly occurs in fvPTC and FTC). These observations were confirmed by the pan-cancer study by Saghafinia et al. who found a significant association between NRAS mutation and hypermethylation events and between BRAF mutation and hypomethylation events in thyroid cancer (Saghafinia et al. 2018). Interestingly, although RAS mutations are common in many types of tumors such as lung adenocarcinoma and prostate cancer this genetic–epigenetic relationship was not detected in other cancers or even in melanomas that mostly harbor NRAS mutations such as thyroid cancer. Therefore, an association between DNA hypermethylation and a specific RAS isoform could be discarded. The BRAF(V600E) mutation is also frequent in colorectal cancer and melanoma, but there is no association between this mutation and hypomethylation events. Conversely, BRAF mutation is strongly associated with hypermethylation events in colorectal cancer (Weisenberger et al. 2006, Cancer Genome Atlas Research Network 2012, Saghafinia et al. 2018). Specifically, in colorectal cancer, BRAF(V600E) has been associated with the so-called ‘CpG island methylator phenotype’ (CIMP) (including tumors that exhibit an exceptionally high frequency of the hypermethylation of some CGIs) (Toyota et al. 1999). From studies done in melanoma, there are some controversial results, but most studies do not find any significant association between BRAF mutation and hyper- or hypomethylations (Lauss et al. 2015, Saghafinia et al. 2018).

Altogether, these findings show a cancer type-specific relationship between BRAF or RAS mutations and aberrant DNA methylation. However, whether these events are dependent on one another requires further studies in controlled experimental systems (e.g., cell lines, mouse models). In this regard, in BRAF-mutated colorectal tumors,
it has been reported that MAFG mediates hypermethylations by binding to target gene promoters and recruiting a corepressor complex that includes DNMT3B (Fang et al. 2014). In thyroid cancer, there are no studies focusing on this genetic-epigenetic relationship. Hou et al. knocked down BRAF in two PTC-derived cell lines by shRNA and found numerous hypermethylated and underexpressed genes, suggesting that these genes were hypomethylated and overexpressed in the presence of BRAF(V600E) and thus pointing out a causal relation (Hou et al. 2011). However, further investigation is required.

**DNA methylation and the BRAF-like and RAS-like phenotypes**

Together, these results highlight the association of DNA methylation profiles with histology and mutations in the BRAF and RAS genes, at least for DTC. However, the relationship between histology, genotype and methylome does not fit perfectly (Fig. 5). For example, fvPTCs harbor more hypermethylations than hypomethylations, except for a subset of fvPTCs that harbor more hypomethylations than hypermethylations; some PTCs that do not contain mutations in BRAF or RAS exhibit more hypermethylations than hypomethylations, while others exhibit more hypomethylations than hypermethylations. This can be resolved with the BRAF-like and RAS-like phenotypes defined by the Cancer Genome Atlas Research Network (Cancer Genome Atlas Research Network 2014). The Cancer Genome Atlas Research Network developed a scoring system based on the expression of 71 genes that classifies PTCs into two groups called BRAF-like and RAS-like tumors depending on whether their gene expression profile more closely resembles BRAF-mutated tumors or RAS-mutated tumors. The Cancer Genome Atlas Research Network shows that these two groups of tumors are different at the genetic and epigenetic levels (Fig. 5), resulting in a different expression program that activates different pathways. BRAF-like tumors are characterized by the overactivation of the MAPK/ERK pathway (preferentially via BRAF), while RAS-like tumors exhibit concurrent activation of the PI3K/AKT and MAPK/ERK pathways (the latter of which is activated at a lower level than that in BRAF-like tumors and preferentially via RAF1 (also known as C-Raf)). In the previous examples, if we take into account the BRAF-like and RAS-like phenotypes, we can see that most fvPTCs are RAS-like and harbor more hypermethylations than hypomethylations, but the subset of fvPTC that is BRAF-like (with or without BRAF mutation) exhibits more hypomethylations than hypermethylations; those tumors that do not contain mutations in

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**Figure 5**

Genetic and epigenetic alterations associated with DTC according to histology, BRAF and RAS mutational state and BRAF-like and RAS-like phenotypes.

1Genetic alterations were considered highly recurrent when present in >5% tumors based on Cancer Genome Atlas Research Network data. 2Genetic alterations were considered lowly recurrent when present in <5% tumors based on Cancer Genome Atlas Research Network data. 3Hyper, more hypermethylations than hypomethylations; hypo, more hypomethylations than hypermethylations. 4FTC data from Yoo et al. (2016). cPTC, classical PTC; FTC, follicular thyroid cancer; tcPTC, tall cell PTC; fvPTC, follicular variant of PTC.

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BRAF or RAS exhibiting more hypomethylations than hypermethylations are BRAF-like while those exhibiting more hypermethylations than hypomethylations are RAS-like. This is in agreement with the study from Chen et al., although they do not specifically use the BRAF-like and RAS-like terms (Chen et al. 2017). These findings suggest that hypo- and hypermethylation events may be downstream of the pathways overactivated in the BRAF-like and RAS-like phenotypes, respectively. Further investigation should be conducted in this area.

**Global DNA hypomethylation in thyroid cancer**

A wide range of techniques to analyze global DNA methylation have been developed (Fig. 1 and Supplementary Table 1) (reviewed in Jordà & Peinado 2010, Toraño et al. 2012), but there are some key points to take into account when interpreting results that are summarized in the Supplementary material. As explained above, global DNA hypomethylation is a common epigenetic feature of cancer. Interestingly, in many cancer types, the degree of global DNA hypomethylation is strongly associated with the tumor grade and stage, which has attracted great interest for its potential clinical use. Nevertheless, little is known about global DNA hypomethylation in thyroid cancer. As shown in Table 2, as far as we know, a total of nine studies have assessed global DNA methylation levels in thyroid tumors and report conflicting results that may be partly explained by the low number of samples included in some studies and the different methods used.

Five of the studies used techniques based on repetitive sequences. They revealed that LINE-1 elements of normal and tumoral samples did not show different levels of DNA methylation (Chalitchagorn et al. 2004, Lee et al. 2008, Keelawat et al. 2015), while Alu elements were slightly hypomethylated in PTC and FTC (Buj et al. 2016). A deeper study of the hypomethylation of Alu elements showed that it occurred in distant metastatic DTC, PDTC and ATC but not in low-risk DTC and pediatric PTC (Klein Hesselink et al. 2018), suggesting the involvement of global hypomethylation of Alu elements

**Table 2** Summary of studies in thyroid cancer analyzing global DNA methylation.

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Method</th>
<th>Global methylation</th>
<th>Discovery series (n/P)</th>
<th>Result</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>COBRA-LINE1</td>
<td>Entire genome</td>
<td>NT (7); PTC (7)</td>
<td>No differences</td>
<td>Chalitchagorn et al. (2004)</td>
</tr>
<tr>
<td>2</td>
<td>IHC with 5mC antibody</td>
<td>Entire genome</td>
<td>NT (9); NG (1); PTC (3); fvPTC (1); FTC (2)</td>
<td>Global hypomethylation in tumors</td>
<td>de Capoa et al. (2004)</td>
</tr>
<tr>
<td>3</td>
<td>IHC with 5mC antibody</td>
<td>Entire genome</td>
<td>NT (17); NG (19); HCA (10); FA (16); FTC (17); FTC (6)</td>
<td>Global hypomethylation in PTC and FTC</td>
<td>Galusca et al. (2005)</td>
</tr>
<tr>
<td>4</td>
<td>LINE-1 pyrosequencing/ LUMA</td>
<td>Compartment</td>
<td>NT (21); FTC (21)</td>
<td>No differences</td>
<td>Lee et al. (2008)</td>
</tr>
<tr>
<td>5</td>
<td>ELISA with 5mC antibody</td>
<td>Entire genome</td>
<td>NT (10); NG (24)</td>
<td>No differences</td>
<td>Brown et al. (2014)</td>
</tr>
<tr>
<td>6</td>
<td>COBRA-LINE1/IHC with 5mC antibody</td>
<td>Compartment/ entire genome</td>
<td>NT (50); FA (15); PTC (17); FTC (18)</td>
<td>No differences in LINE-1 methylation but global hypermethylation in tumors</td>
<td>Keelawat et al. (2015)</td>
</tr>
<tr>
<td>7</td>
<td>QUAlu</td>
<td>Compartment</td>
<td>NT (9); cPTC (31); FTC (14)</td>
<td>Global Alu hypomethylation in PTC and FTC</td>
<td>Buj et al. (2016)</td>
</tr>
<tr>
<td>8</td>
<td>QUAlu</td>
<td>Compartment</td>
<td>NT (20); PTC (40); FTC (21); PDTC (7); ATC (9); M1 (24); pediatric FTC (13)</td>
<td>Global Alu hypomethylation in distant metastatic PTC and distant metastatic FTC as well as the paired M1, and in PDTC and ATC</td>
<td>Klein Hesselink et al. (2018)</td>
</tr>
<tr>
<td>9</td>
<td>ELISA with 5mC antibody</td>
<td>Entire genome</td>
<td>Blood: controls (6); PTC (12)</td>
<td>No differences</td>
<td>Ceolin et al. (2018)</td>
</tr>
</tbody>
</table>

*Part of the genome in which global DNA methylation has been analyzed. *DNA methylation was assessed in postsurgical tissue unless otherwise stated. 5mC, 5-methylcytosine; ATC, anaplastic thyroid cancer; COBRA, combined bisulfite restriction analysis; cPTC, classical PTC; FA, follicular adenoma; fPTC, follicular variant of PTC; FTC, follicular thyroid cancer; IHC, immunohistochemistry; HC, Hurthle cell adenoma; LUMA, luminometric methylation assay; M1, distant metastasis; NG, nodular goiter; NT, normal tissue; PDTC, poorly differentiated thyroid cancer; PTC, papillary thyroid cancer; QUAlu, quantification of unmethylated Alu.
in thyroid cancer progression and dedifferentiation. This is in agreement with studies in other cancer types, such as hepatocellular carcinoma or cervical cancer, in which global hypomethylation correlates with disease progression (Lin et al. 2001, Yegnasubramanian et al. 2008). These findings in thyroid cancer may have important prognostic applications, especially in preoperative fine-needle aspiration biopsies (FNAB), which would help in treatment planning. The differences between the results about LINE-1 and Alu elements could be explained by the fact that the studies analyzing LINE-1 elements included FA, PTC and FTC but did not include aggressive tumors. We cannot discard the use of different techniques with different sensitivity and accuracy as being responsible for the different results. On the other hand, as explained in the Supplementary material, these apparently opposite findings are biologically plausible. In this regard, the different methylation between LINE-1 and Alu elements also occurs in other cancers (Benard et al. 2013, Park et al. 2014).

Another interesting result from these studies was that while global methylation of LINE-1 elements varied between different normal tissues, especially in the normal thyroid, all normal tissues displayed similar levels of unmethylated Alu elements (Chalitchagorn et al. 2004, Buj et al. 2016). Conversely, tumors showed a broad variation of the DNA methylation of both LINE-1 and Alu elements. For example, colon and lung cancer exhibited 2- to 3-fold higher levels of unmethylated Alu elements than thyroid cancer. On the other hand, the Alu hypomethylation was similar between distant metastases and matched primary tumors, suggesting that Alu methylation remained stable during metastatic spread in thyroid cancer (Klein Hesselink et al. 2018).

Four more studies used antibodies that recognize 5mC to evaluate global hypomethylation in thyroid cancer, and three of them were based on immunohistochemistry while one used ELISA. The analyses of benign lesions (hyperplasia, FA and Hurthle adenoma) did not find differences between benign lesions and normal thyroid tissues (Galusca et al. 2005, Brown et al. 2014, Keelawat et al. 2015), except for de Capoa et al., but they only analyzed one sample (de Capoa et al. 2004). Thus, global DNA hypomethylation in thyroid cancer does not seem to be an early event as described in other cancer types (Ehrlich 2009). The results on global DNA hypomethylation in DTC were more variable. de Capoa et al. and Galusca et al. showed global hypomethylation in malignant tumors compared to normal tissues (de Capoa et al. 2004, Galusca et al. 2005). In contrast, Keelawat et al. did not find global hypomethylation but rather found global hypermethylation (Keelawat et al. 2015). These inconsistent results are probably due to technical issues that mainly include the use of different antibodies with different sensitivities. In this regard, the two studies showing global hypomethylation used the same antibody. Although further investigation is needed, these results suggest the diagnostic potential of global DNA methylation measured by specific antibodies. Moreover, the fact that benign lesions do not display global alterations is very promising, especially for thyroid nodules with indeterminate cytology.

Only one of the studies analyzed the relationship between global hypomethylation and mutations in BRAF and RAS, and it found an association in distant metastatic DTC but not in low-risk DTC (Klein Hesselink et al. 2018). Specifically, BRAF-mutated distant metastatic DTC showed hypomethylation of the Alu elements, but RAS-mutated tumors did not. Interestingly, distant metastatic DTC harboring no mutations in BRAF or RAS showed notable variability, which could reflect the BRAF-like and RAS-like phenotypes.

Several studies indicate that the global DNA methylation in peripheral blood leukocytes differs significantly between healthy individuals and patients with different cancer types (Li et al. 2012). There is only one study analyzing global DNA methylation in blood from PTC patients and control individuals (using a 5mC DNA ELISA), and they did not find differences (Ceolin et al. 2018).

Altogether, these findings indicate that global hypomethylation plays a role in thyroid tumorigenesis. However, further investigation is required. The analysis of global hypomethylation in different compartments of the genome using different techniques in a large cohort of thyroid samples would provide enlightening insight.

### Focal DNA methylation alterations in thyroid cancer: gene-specific studies

Gene-specific DNA methylation has been broadly studied. Accordingly, many investigators in thyroid cancer have focused on the hypermethylation of specific tumor suppressor genes (TSGs) as an alternative to mutational inactivation (Supplementary Table 2). Some of the most recurrently hypermethylated TSGs in thyroid cancer are Ras association domain family 1, isoform A (RASSF1A), cyclin-dependent kinase inhibitor 2A (CDKN2A or P16INK4A) and death-associated protein kinase1 (DAPK) (Table 3). The RASSF1A gene encodes a signaling protein
<table>
<thead>
<tr>
<th>Study No.</th>
<th>Method</th>
<th>Discovery series</th>
<th>Gene</th>
<th>Study No.</th>
<th>Method</th>
<th>Discovery series</th>
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<td>1</td>
<td>MSP</td>
<td>NT (4), NG (13),</td>
<td>RASSF1A</td>
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<td>qMSP</td>
<td>NT (14), BTLs (9), FTC (12), PTC (30)</td>
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<td>NT (4), NG (13),</td>
<td>RASSF1A</td>
<td>3</td>
<td>M, qMSP</td>
<td>NT (13), NG (20), FA (24), FTC (10), 10, HCTC (9), CL (9)</td>
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<td>M</td>
<td>RASSF1A</td>
<td>4</td>
<td>M, qMSP</td>
<td>M</td>
</tr>
<tr>
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<td>MSP</td>
<td>M</td>
<td>RASSF1A</td>
<td>5</td>
<td>M, qMSP</td>
<td>M</td>
</tr>
<tr>
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<td>M</td>
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<td>qMSP</td>
<td>M</td>
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<tr>
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<td>qMSP</td>
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<td>RASSF1A</td>
<td>12</td>
<td>PCR + MS-RE</td>
<td>M</td>
</tr>
<tr>
<td>12</td>
<td>PCR + MS-RE</td>
<td>M</td>
<td>RASSF1A</td>
<td>13</td>
<td>qMSP</td>
<td>M</td>
</tr>
<tr>
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<td>qMSP</td>
<td>M</td>
</tr>
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<td>qMSP</td>
<td>M</td>
<td>RASSF1A</td>
<td>15</td>
<td>qMSP</td>
<td>M</td>
</tr>
</tbody>
</table>

Table 3: Summary of candidate approach DNA methylation studies on classical tumor suppressor genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Study No.</th>
<th>Method</th>
<th>Discovery series</th>
<th>Potential clinical value</th>
<th>Potential clinical value</th>
<th>Potential clinical value</th>
<th>Potential clinical value</th>
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<tbody>
<tr>
<td>RASSF1A</td>
<td>1</td>
<td>MSP</td>
<td>NT (4), NG (13),</td>
<td>Hypermethylation frequency in aggressive variants.</td>
<td>Hypermethylation frequency in aggressive variants.</td>
<td>Hypermethylation frequency in aggressive variants.</td>
<td>Hypermethylation frequency in aggressive variants.</td>
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<td>RASSF1A</td>
<td>2</td>
<td>qMSP</td>
<td>NT (14), BTLs (9), FTC (12), PTC (30)</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
</tr>
<tr>
<td>RASSF1A</td>
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<td>M, qMSP</td>
<td>M</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
</tr>
<tr>
<td>RASSF1A</td>
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<td>M, qMSP</td>
<td>M</td>
<td>No relationship with BRAF mutation.</td>
<td>No relationship with BRAF mutation.</td>
<td>No relationship with BRAF mutation.</td>
<td>No relationship with BRAF mutation.</td>
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<td>RASSF1A</td>
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<td>qMSP</td>
<td>M</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
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</tr>
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</tr>
<tr>
<td>RASSF1A</td>
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<td>qMSP</td>
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<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
<td>Early event in thyroid tumorigenesis.</td>
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<tr>
<td>RASSF1A</td>
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<td>M</td>
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<td>Early event in thyroid tumorigenesis.</td>
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<td>M</td>
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<tr>
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<td>qMSP</td>
<td>M</td>
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<td>Early event in thyroid tumorigenesis.</td>
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<td>Early event in thyroid tumorigenesis.</td>
</tr>
<tr>
<td>RASSF1A</td>
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<td>PCR + MS-RE</td>
<td>M</td>
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<td>13</td>
<td>qMSP</td>
<td>M</td>
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</tbody>
</table>
### Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Study No.</th>
<th>Method</th>
<th>Discovery series (n)</th>
<th>Methylation status*</th>
<th>Potential clinical value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16/INK4A (CDKN2A)</td>
<td>1</td>
<td>MSP</td>
<td>FA (8), PTC (12), CL (4) NT (15), FA (18) PTC (16), FTC (18), PDTC (12), ATC (13)</td>
<td>NT: M, BTLs: M, DTC: M, PDTC/ATC: M</td>
<td>Higher hypermethylation frequency in aggressive variants. Related to N1 and M1</td>
<td>Elisei et al. (1998) Boltze et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>MSP</td>
<td>NT (15), NG (20), FA (24), PTC (23), fPTC (10), CL (5)</td>
<td>NT: U, BTLs: U, DTC: U, PDTC/ATC: –</td>
<td>No correlation with clinical any parameters</td>
<td>Hoque et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>MSP, qMSP</td>
<td>NT (15), NG (20), FA (24), PTC (23), fPTC (10), CL (5)</td>
<td>NT: U, BTLs: U, DTC: U, PDTC/ATC: –</td>
<td>Higher hypermethylation frequency in aggressive variants</td>
<td>Schagdarsurengin et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>MSP</td>
<td>NG (12), FA (10), PTC (13), FTC (10), ATC (9), CL (9)</td>
<td>NT: –, BTLs: U, DTC: –</td>
<td>Higher hypermethylation frequency in aggressive variants</td>
<td>Related to progression</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>MSP</td>
<td>PTC (39)</td>
<td>NT: –, BTLs: U, DTC: –</td>
<td>Related to progression</td>
<td>No correlation with clinical any parameters</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>COBRA</td>
<td>BTLs (25), PTC (25)</td>
<td>NT: –, BTLs: U, DTC: –</td>
<td>Related to progression</td>
<td>No correlation with clinical any parameters</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>MSP</td>
<td>NG (20), cPTC (27), fPTC (15), cPTC (3)</td>
<td>NT: –, BTLs: U, DTC: –</td>
<td>Related to progression</td>
<td>No correlation with clinical any parameters</td>
</tr>
<tr>
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<td>8</td>
<td>qMSP</td>
<td>NT (15), BTLs (44), cPTC (17), fPTC (10), FTC (7), HCTC (2)</td>
<td>NT: U, BTLs: U, DTC: U</td>
<td>No correlation with clinical any parameters</td>
<td>Brait et al. (2012)</td>
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<tr>
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<td>9</td>
<td>MSP</td>
<td>NT (21), PTC (74)</td>
<td>NT: U, BTLs: U, DTC: –</td>
<td>Related to aggressiveness</td>
<td>Wang et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>qMSP</td>
<td>NT (71), FA (83), HCTC (44), FTC (46), fPTC (42), cPTC (53)</td>
<td>NT: U, BTLs: U, DTC: –</td>
<td>Related to aggressiveness</td>
<td>Stephen et al. (2018)</td>
</tr>
<tr>
<td>DAPK1</td>
<td>1</td>
<td>MSP, qMSP</td>
<td>NT (15), NG (20), FA (24), PTC (23), fPTC (10), CL (5)</td>
<td>NT: M, BTLs: M, DTC: M, PDTC/ATC: –</td>
<td>Higher hypermethylation frequency in cPTC and tCPTC. Related with multifocality</td>
<td>Hu et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>qMSP</td>
<td>cPTC (127), fPTC (82), tcPTC (22)</td>
<td>NT: –, BTLs: M, DTC: M</td>
<td>Higher hypermethylation frequency in cPTC and tCPTC. Related with multifocality</td>
<td>Related to progression</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>MSP</td>
<td>NG (12), FA (10), PTC (13), FTC (10), ATC (9), CL (9)</td>
<td>NT: –, BTLs: U, DTC: U</td>
<td>Higher hypermethylation frequency in aggressive variants</td>
<td>Stephen et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>qMSP</td>
<td>NT (15), BTLs (44), cPTC (17), fPTC (10), FTC (7), HCTC (2)</td>
<td>NT: M, BTLs: M, DTC: M</td>
<td>Higher hypermethylation frequency in aggressive variants</td>
<td>Stephen et al. (2018)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>qMSP</td>
<td>HCTC (26), FTC (27)</td>
<td>NT: –, BTLs: U, DTC: –</td>
<td>Higher hypermethylation frequency in aggressive variants</td>
<td>Stephen et al. (2018)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>qMSP</td>
<td>NT (71), FA (83), HCTC (44), FTC (46), fPTC (42), cPTC (53)</td>
<td>NT: U, BTLs: U, DTC: U</td>
<td>Higher hypermethylation frequency in aggressive variants</td>
<td>Stephen et al. (2018)</td>
</tr>
</tbody>
</table>

*Methylation status of gene promoter: M, methylated; U, unmethylated; hyper, hypermethylated compared to normal tissue.

ATC, anaplastic thyroid cancer; BTLs, benign thyroid lesions; CL, cell lines; COBRA, combined bisulfite restriction analysis; cPTC, classical PTC; FA, follicular adenoma; FTC, follicular thyroid cancer; fPTC, follicular variant of PTC; HCTC, Hurthle cell thyroid cancer; HTT, hyalinizing trabecular tumor; MSP, methyl-sensitive PCR; MS-RE, methylation-sensitive restriction enzyme; NG, nodular goiter; NT, normal tissue; PDTC, poorly differentiated thyroid cancer; PTC, papillary thyroid cancer; qMSP, quantitative MSP.
containing a Ras association domain and is involved in multiple apoptotic and cell cycle checkpoint pathways. The main mechanism of RASSF1A inactivation, a frequent event in many cancers, appears to be through promoter methylation rather than mutational events (Dammann et al. 2000, Agathangelou et al. 2005). Schagdarsurengin et al. found for the first time that RASSF1A was hypermethylated in thyroid cancer with a slightly higher frequency in more aggressive histotypes (Schagdarsurengin et al. 2002). Many other studies confirmed RASSF1A hypermethylation in thyroid tumors, including a recent meta-analysis, although most results revealed a considerable overlap in methylation levels between benign and malignant tumors (Nakamura et al. 2005, Hou et al. 2008, Mohammadi-asl et al. 2011, Stephen et al. 2011, Niu et al. 2017) (Table 3), suggesting that RASSF1A hypermethylation may be an early epigenetic event in thyroid carcinogenesis (Xing et al. 2004, Brown et al. 2014). Different studies have shown the potential of RASSF1A hypermethylation as a biomarker of aggressive tumors, while other authors have failed to find any relationship between RASSF1A hypermethylation and prognostic factors (Schagdarsurengin et al. 2006, Mohammadi-asl et al. 2011, Braït et al. 2012, Niu et al. 2017). Thus, further studies are required in larger series of samples using more quantitative techniques. P16INK4A is a cell cycle regulator that induces G1 phase arrest, whose functional loss is frequent in cancer. Mutations in this gene are rarely observed in primary thyroid tumors (Calabrò et al. 1996, Yane et al. 1996), whereas promoter hypermethylation, which causes gene silencing, is quite common (Table 3). Many studies, including a meta-analysis based on 17 case-control studies (804 thyroid cancer patients, 487 controls), confirmed the significantly higher frequency of P16INK4A hypermethylation in thyroid cancer than in normal samples (Boltze et al. 2003, Schagdarsurengin et al. 2006, Meleck et al. 2007, Zafon et al. 2008, Wu et al. 2015). However, its usefulness as a prognostic marker remains questionable, as summarized in Table 3. DAPK1, which belongs to the DAPK family of calcium/calmodulin-dependent kinases, participates in many cellular processes such as apoptosis, autophagy, and cell survival, and is also involved in cancer (reviewed in Farag & Roh 2019). DAPK1 promoter hypermethylation has been associated with an increased risk of developing cancer and poor prognosis in several cancer types (Dai et al. 2016, Qi & Xiong 2018, Yang et al. 2018). As shown in Table 3, DAPK1 is hypermethylated in benign and malignant thyroid tumors but the clinical value of DAPK1 hypermethylation is not well established.

In addition, the methylation of thyroid-specific genes, such as thyroid-stimulating hormone receptor (TSHR) or NIS, has also been extensively investigated in thyroid cancer (Table 4 and Supplementary Table 2). TSHR plays a central role in the regulation of thyroid growth and function. Somatic TSHR mutations have been found in both benign and malignant thyroid neoplasms (Davies et al. 2010), but the involvement of TSHR genetic status in thyroid carcinogenesis is not clear. A recent study showed that TSHR mutations may be associated with an increased cancer risk when present at high allelic frequency (Mon et al. 2018). In thyroid cancer, TSHR expression is also repressed by aberrantly methylation of gene promoter (Table 4). However, several studies found TSHR methylated in benign lesions (Hoque et al. 2005, Schagdarsurengin et al. 2006, Braït et al. 2012, Kartal et al. 2015, Stephen et al. 2018), which limits the discriminatory power for diagnostic purposes. Interestingly, TSHR methylation has been found inversely associated with tumor recurrence (Smith et al. 2007). NIS is a transmembrane glycoprotein that mediates the active transport of iodide from the bloodstream into the follicular thyroid cells and is mainly regulated by the thyroid-stimulating hormone (TSH). The role of NIS is key for effective diagnosis and treatment of thyroid cancer since RAI accumulation is primarily mediated by NIS. Accordingly, the decreased NIS expression and/or impairment in NIS plasma membrane trafficking (De la Vieja & Santisteban 2018) are well demonstrated factors showing poor prognosis in thyroid cancer. However, the relationship between NIS and thyroid cancer is complex and not well understood (de Morais et al. 2018). Mutations in the NIS gene do not appear to be a major cause for reduced NIS expression/function in thyroid cancer (Russo et al. 2001). In contrast, many studies have reported the methylation of NIS promoter although results are controversial (Table 4). Interestingly, Galrao et al. identified a distal enhancer that was hypermethylated in DTC regulating NIS expression (Galrao et al. 2014). This new finding provides a basis for further investigation in the epigenetic regulation of NIS.

### Focal DNA methylation alterations in thyroid cancer: genome-wide studies

In addition to validating results from candidate approach studies, genome-wide DNA methylation studies have allowed the identification of novel differentially methylated sequences that may regulate the expression of genes involved in thyroid cancer tumorigenesis (Table 1). In this regard, Rodríguez-Rodero et al. showed...
that in ATC, the membrane-associated protein 17 (MAP17) gene was hypomethylated in its promoter region and overexpressed compared to normal tissues. They showed that overexpression of MAP17 induced tumor growth in vitro and in vivo (Rodriguez-Rodero et al. 2013). Zhang et al. identified 14 novel genes regulated by DNA methylation in PTC (Table 1) that were used to construct a core cofunction network that revealed the potential of the C-X-C motif chemokine ligand (CXCL12), a chemokine involved in the immune response, as a key player in thyroid tumorigenesis (Zhang et al. 2017). Moreover, the expression levels of these 14 genes gave the ability to discriminate between PTC patients and healthy individuals. By integrating DNA methylation and transcriptomic data, Beltrami et al. found 185 genes with a negative correlation between methylation and expression that mostly affected fibroblast growth factor (FGF) and retinoic acid (RA) signaling pathways (Beltrami et al. 2017). Other interesting hypomethylated genes that were identified in genome-wide DNA methylation analyses were high-mobility group box 2 (HMGB2), which may play a role in PTC cell proliferation, and FYVE, RhoGEF and PH domain-containing 1 (FDG1), which may be involved in cell invasion (Hou et al. 2011). Additionally, Lin et al. identified HORMA domain-containing 2 (HORMAD2) and showed that its hypermethylation and repression induced the progression of thyroid cancer, while its hypomethylation and overexpression retarded cell growth and mobility and facilitated apoptosis (Lin et al. 2018).

From a translational point of view, genome-wide DNA methylation studies are an important source of new biomarkers to develop algorithms and tools with diagnostic and prognostic value. For example, Mancikova et al. identified two putative biomarkers associated with recurrence-free survival, etoposide-induced 2.4 (E124) and Wilms’ tumor 1 (WT1) (Mancikova et al. 2014). They also found kallikrein 10 (KLK10) to be hypomethylated and overexpressed in BRAF-mutated tumors. Further analyses based on KLK10 allowed the development of an algorithm related to BRAF- and RAS-like phenotypes with prognostic implications in thyroid cancer (Buj et al. 2018). On the other hand, Bisarro dos Reis et al. developed a prognostic algorithm based on 21 CpGs able to predict recurrence in DTC with high specificity but low sensitivity (Bisarro dos Reis et al. 2017). However, the series of samples used contained a low number of recurrent cases; thus, further analyses are required to validate its potential for prognostic use. Finally, Yim et al., who profiled PTC DNA methylation by RRBS, developed a new diagnostic method, the so-called diagnostic DNA methylation signature (DDMS) approach, which is based on 373 differentially methylated regions with tissue-specific DNA methylation patterns in benign and malignant nodules (Yim et al. 2019). A notable proportion of these markers were associated with active enhancers and cancer-related genes. Importantly, the DDMS approach distinguishes benign from malignant nodules with high sensitivity and specificity and thus has the potential to provide outstanding diagnostic accuracy for thyroid nodules, which may decrease overdiagnosis and unnecessary thyroidectomies.

DNA methylation as a therapeutic target in thyroid cancer

As explained, the aberrant methylation of DNA can play a key role in tumorigenesis. In addition, DNA methylation is inherently reversible, which makes targeted therapies against it very attractive for cancer treatment. Therefore, much effort has been made to study the potential of drugs that inhibit this type of epigenetic modification to induce the re-expression of silenced genes in different malignancies. Over the past few decades, different demethylating drugs have been developed and tested in different human neoplasms. There are two different classes of demethylating agents: nucleoside DNMT inhibitors and non-nucleoside DNMT inhibitors. Treatment with these agents causes a reduction in global DNA methylation rather than demethylation in specific regions (reviewed in Mani & Herceg 2010).

The most commonly used demethylating agents are the first ones described: 5-azacytidine (azacitidine, AZA) (Sorm et al. 1964) and 5-aza-2’-deoxycytidine (decitabine, DAC), both of which are nucleoside DNMT inhibitors. Around 1970, clinical trials in Europe and the United States using AZA began focusing on the treatment of both solid and blood neoplasms (Sorm & Vesely 1968). The results showed the effectiveness of treating patients with acute myeloid leukemia (AML) resistant to conventional treatment and/or with relapse with AZA and DAC. In contrast, no significant responses were found in other types of blood cancers or in solid tumors to those drugs. At that time, the US Food and Drug Administration (FDA) did not approve AZA due to its high levels of toxicity relative to its antitumor efficacy. Nearly 40 years later, in 2004, after adjusting the dosage to reduce toxicity and increase efficiency, it was approved for clinical use to treat myelodysplastic syndromes (MDS) (Kaminskas et al. 2005).
In 2006, DAC was also approved for the treatment of MDS (Kantarjian et al. 2006). More recently, other agents have been identified such as zebularine or procaine, and their potential use in demethylating therapy is being tested (Villar-Garea et al. 2003, Marquez et al. 2005, Mani & Herceg 2010).

Curiously, demethylating agents are more effective in treating hematologic cancers than solid tumors despite the large amount of evidence showing that aberrant DNA methylation is a trait common to all tumorigenic processes (Sharma et al. 2010). Such trouble in accomplishing therapeutic effectiveness could be due

### Table 4  Summary of candidate approach DNA methylation studies on thyroid-specific genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Study No.</th>
<th>Method</th>
<th>Discovery series (n)*</th>
<th>Methylation statusb</th>
<th>Potential clinical value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSHR</td>
<td>1</td>
<td>MSP, qMSP</td>
<td>FA (8), PTC (39), FTC (15), ATC (11), CL (6)</td>
<td>NT – U hyper hyper</td>
<td>Diagnostic marker</td>
<td>Xing et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>MSP</td>
<td>NG (12), FA (10), PTC (13), FTC (10), ATC (9), CL (9)</td>
<td>M M M –</td>
<td></td>
<td>Schagdarsurengin et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>MSP</td>
<td>NT(2), NG (15), FA (10), PTC (30)</td>
<td>U M hyper –</td>
<td>Inverse correlation with recurrence</td>
<td>Smith et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>qMSP</td>
<td>NT (71), FA (83), cPTC (53), fvPTC (42), HCTC (44), FTC (46)</td>
<td>M M M –</td>
<td></td>
<td>Stephen et al. (2018)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>qMSP</td>
<td>NT (15), NG (20), FA (24), PTC (23), fvPTC (10), CL (5)</td>
<td>M M M –</td>
<td></td>
<td>Hoque et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>qMSP</td>
<td>NT (15), BTLs (44), cPTC (17), fVPTC (10), FTC (7), HCTC (2)</td>
<td>M M hyper –</td>
<td></td>
<td>Brait et al. (2012)</td>
</tr>
<tr>
<td>NIS (SLC5A5)</td>
<td>1</td>
<td>MSP</td>
<td>NT(2), NG (15), FA (10), PTC (30)</td>
<td>U U hyper –</td>
<td>No prognostic factor</td>
<td>Smith et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>MS-MLPA</td>
<td>NT (5), NG (3), PTC (11), FTC (2)</td>
<td>M M M –</td>
<td>Early event</td>
<td>Stephen et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>MSP</td>
<td>NT (30), BTLs (10), PTC (18), FTC (2)</td>
<td>M M M –</td>
<td></td>
<td>Galrão et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>BS-sequencing</td>
<td>NT (30), BTLs (10), PTC (18), FTC (2)</td>
<td>M M hyper –</td>
<td>Correlation with expression</td>
<td>Galrão et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>MSP, BS-sequencing</td>
<td>NT (24), PTC (24)</td>
<td>M – hyper –</td>
<td>Related to BRAF(V600E)</td>
<td>Choi et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>qMSP</td>
<td>HCTC (26), FTC (27)</td>
<td>– – M –</td>
<td>No differences between HCTC and FTC</td>
<td>Stephen et al. (2015)</td>
</tr>
</tbody>
</table>

*DNA methylation was assessed in postsurgical tissue unless otherwise stated. bMethylation status of gene promoter: hyper, hypermethylated compared to normal tissue; M, methylated; U, unmethylated. cMethylation status of NIS enhancer.

ATC, anaplastic thyroid cancer; BS-sequencing, bisulfite sequencing; BTLs, benign thyroid lesions; CL, cell lines; cPTC, classical PTC; FA, follicular adenoma; FNAB, fine-needle aspiration biopsy; FTC, follicular thyroid cancer; fvPTC, follicular variant of PTC; HCTC, Hurthle cell thyroid cancer; MSP, methyl-sensitive PCR; NG, nodular goiter; PTC, papillary thyroid cancer; qMSP, quantitative MSP.
to a variety of reasons, such as lower DNMT activity in solid tumors (Lin et al. 2009) or that the starting level of aberrant methylation in hematological malignancies is higher than that in solid tumors (Issa et al. 1997). Another limitation is that these agents need actively dividing cells to take action. Therefore, slow-growing tumors might require longer dosing schedules due to their short half-life or improvement in drug delivery and plasma stability (Howell et al. 2010).

It may seem that the encouraging effects seen in trials to treat hematological malignancies and preclinical data on solid tumors will never reach a clinical application. However, several studies have shown the association between the overexpression of DNMTs and chemoresistance (Wang et al. 2001, Qiu et al. 2002, Segura-Pacheco et al. 2006), and the treatment of cancer cell lines with DNMT inhibitors can revert this resistance to therapy (Qiu et al. 2005). In light of these findings, clinical trials with promising results have been conducted to test whether demethylating drugs can enhance susceptibility to other therapies when administered in combination, especially in resistant tumors (Linnekamp et al. 2017).

Thyroid cancer is not an exception to all the details explained above. Initially, in vitro studies focused on the ability of demethylating drugs to restore the expression of different genes to sensitize thyroid cancer cells to RAI treatment. Venkataraman et al. reported an increase in the mRNA and gene expression of NIS in thyroid cancer cell lines treated with AZA compared to those observed without AZA treatment. The increased expression of the NIS gene was correlated with an increase in RAI uptake in some of the cell lines (Venkataraman et al. 1999). Nevertheless, other similar studies could not show a significant increase in RAI uptake when different thyroid cell lines were treated with AZA or DAC, highlighting that the mechanism may depend on the methylation and differentiation status of the cell (Tuncel et al. 2007, Miasaki et al. 2008). Although significant cell redifferentiation is not achieved, DAC and zebularine are able to inhibit cell proliferation and migration in thyroid cancer cell lines (Miasaki et al. 2008, Kim et al. 2013).

There have only been two clinical trials focusing on the treatment of thyroid cancer patients with demethylating agents to sensitize tumors to RAI (ClinicalTrials.gov Identifier: NCT00085293 and NCT00004062). Both included patients with recurrent and/or metastatic DTC that were resistant to RAI. Unfortunately, no partial or complete responses were observed, while treatment caused serious side effects. Therefore, as with other types of malignancies, efforts have also been made toward exploring the potential of these agents in combination with other treatments. Significant redifferentiation accompanied by growth inhibition and cell apoptosis was observed when cells were treated with DAC and RA. However, no increase in RAI uptake was observed due to the cytoplasmic localization of the NIS protein (Vivaldi et al. 2009). Other studies use demethylating drugs to increase the sensitivity of thyroid cancer cells to other agents such as TNF-related apoptosis-inducing ligand (TRAIL), which induces apoptosis (Siraj et al. 2011) or to upregulate immune-related genes in cancer cells to enhance their response to cancer immunotherapies (Gundu et al. 2013, 2014). A strong synergistic effect was also seen when DAC was combined with everolimus (an mTOR inhibitor) to treat thyroid cancer cells, opening a promising scenario to overcome drug resistance (Vitale et al. 2017). However, the most explored and promising option, not just in thyroid cancer, is the combination of demethylating agents with HDAC inhibitors such as trichostatin A (TSA), sodium butyrate or valproic acid. In vitro, this combination can restore NIS transcription to levels approaching those present in RAI-responder tumors (Li et al. 2007) and even increase RAI uptake (Provenzano et al. 2007, Massimino et al. 2018). In addition, they can also inhibit cell growth and invasion (Mitmaker et al. 2011).

Finally, it is important to mention the role that demethylating agents have been playing through the years as important tools to discover and study new prognostic and diagnostic biomarkers (Murgo 2005, Zuo et al. 2010, Latini et al. 2011, Moraes et al. 2016, Wu et al. 2016, Cao et al. 2018).

In conclusion, there is little evidence of the effectiveness of demethylating agents in thyroid cancer. Most studies have tested these drugs in a variety of cancer cell lines obtaining promising results that have not been translated into clinical practice. However, despite the unsuccessful results in clinical trials with their use as solo agents, they may be a potentially useful therapy when combined with other drugs.

**DNA methylation in thyroid cancer cell lines**

Established human thyroid cancer cell lines are the most widely used models to study thyroid tumorigenesis, including studies aimed at understanding the DNA...
methylation landscape. However, it has been shown that cell lines derived from DTC, both PTC and FTC, display mRNA expression profiles closer to dedifferentiated \textit{in vivo} thyroid tumors (i.e., ATC) than to differentiated ones (van Staveren et al. 2007, Saiselet et al. 2012). This can be explained by the prior selection of initiating cells and the \textit{in vitro} evolution of the cell lines. Interestingly, some of the genes commonly upregulated in ATC and thyroid cancer cell lines are related to DNA replication, which is in accordance with their high proliferation rate.

Considering that DNA methylation is involved in the regulation of gene expression, how does cellular immortalization affect DNA methylation in thyroid cancer cell lines? Although there are few studies profiling DNA methylation in thyroid cancer cell lines, they note that DNA methylation follows a similar pattern as gene expression. Rodero-Rodríguez et al. analyzed four cell lines (one derived from PTC, one from FTC, one from ATC and one from MTC), and all of them exhibited methylomes that more closely resembled undifferentiated tumors than differentiated ones (Rodriguez-Rodero et al. 2013). Typically, immortalized cell lines exhibit hypermethylation (Smiraglia et al. 2001). However, this is not the case for thyroid cancer cell lines, which, based on the study by Rodero-Rodriguez et al., show more hypomethylation than hypermethylation events (Rodriguez-Rodero et al. 2013). This result is in agreement with Klein Hesselink et al. who found that the global hypomethylation level of Alu elements in PTC- and FTC-derived cell lines was similar to that observed in ATC-derived cell lines and \textit{in vivo} PDTC and ATC samples (Klein Hesselink et al. 2018).

However, some gene-specific DNA methylation studies found a good agreement between \textit{in vivo} DTC tumors and cell lines. Therefore, despite the limitations of the use of cell lines, they provide a good model for controlled experiments, for example, to study the DNA methylation-mediated regulation of candidate genes identified by genome-wide studies or to investigate the effect of specific treatments, such as the ability of demethylating drugs to restore the expression of different genes to sensitize thyroid cancer cells to RAI.

Altogether, these studies indicate that thyroid cancer cell lines are an important tool for thyroid cancer research, but the differences in gene expression and DNA methylation compared to \textit{in vivo} tumors should be taken into account when extrapolating results obtained from these cells.

**Concluding remarks and perspectives**

Numerous studies on DNA methylation in thyroid cancer have improved our understanding of thyroid carcinogenesis. However, we still do not have a complete picture of the methylation landscape, especially for histological subtypes other than PTC. The huge catalog of DNA methylation alterations, the association of DNA hypomethylation with cancer progression and dedifferentiation, the existence of different methylomes related to different clinical and molecular phenotypes and the influence of immune-infiltrating cells in tumor DNA methylation patterns are some of the recent findings that will most likely define the direction of future research in the field of DNA methylation in thyroid cancer. In addition, numerous studies confirm the importance of DNA methylation as a source of novel biomarkers in thyroid cancer. Indeed, some studies propose potential diagnostic and prognostic markers, although the combination of DNA methylation alterations with other epigenetic and/or genetic alterations may improve their clinical value. Finally, DNA methylation is also a fundamental area of interest from a therapeutic perspective. Therefore, further \textit{in vitro} and \textit{in vivo} functional experiments to better understand the implications and underlying mechanisms of DNA methylation alterations in thyroid cancer as well as the evaluation of candidate biomarkers through case–control studies and prospective trials are warranted.

**Supplementary data**

This is linked to the online version of the paper at https://doi.org/10.1530/ERC-19-0093.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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