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Chapter

Research Progress of DNA Methylation in Thyroid Cancer

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Abstract

We have summarized increasing data from all kinds of experiment results of papers in recent years, which are associated with tumor suppressor genes, oncogenes, and thyroid-specific genes and attempt to elucidate the importance of epigenetic modifications and the mechanisms of aberrant DNA methylation in thyroid cancer in this review. The results showed that current articles have revealed the importance of epigenetic modifications and the different types of mechanisms in thyroid cancer. The mechanisms of DNA methylation related to thyroid cancer demonstrate that acquired epigenetic abnormalities together with genetic changes play an important role in alteration of gene expression patterns. Aberrant DNA methylation has been well known in the CpG regions. Among the genes identified, we have shown the status of DNA promoter methylation in papillary, follicular, medullary, and anaplastic thyroid cancer. It suggested that thyroid cancer subtypes present differential promoter methylation signatures, which will encourage potential thyroid cancer detection in its early stages, assessment of prognosis, and targeted cancer treatment.

Keywords: thyroid carcinoma, DNA methylation, epigenetic inheritance, tumor suppressor genes, oncogene genes, thyroid-specific genes

1. Introduction

Thyroid cancer is the most frequent endocrine neoplasia. The National Cancer Institute estimated that there would be 44,670 new cases of thyroid cancer (TC) with 1690 deaths in 2010, and with an overall estimate of 56,870 new cases by 2017, and its incidence has been increasing in recent decades. Compared with other adult cancers, TC tends to occur in younger people between the ages of 20 and 60. It is three times more common in women than men [1] and has the fastest rising incidence rates in women and the second fastest in men with an annual percentage change of approximately 5%, making TC the sixth most common cancer in women [2]. There are four main types of which papillary and follicular (PTC, FTC) types together account for >90% followed by medullary thyroid cancers (MTC) with 3–5% and anaplastic carcinomas (ATC) making up <3% [3]. Reasons for this trend have been attributed to improvement in imaging (ultrasound technology) that is allowing the identification of ever smaller thyroid nodules. However, with this gain in detection, determining which benign nodules (adenomas) will progress to cancer cannot be determined on the basis of histology alone, underscoring the need for

genetic markers of early detection for TC. Recently, epigenetic alterations have been shown to play a role in the development and progression of thyroid cancer.

With the deepening of tumor research, it has been gradually found that epigenetics plays an important role in the occurrence and development of tumors. Mechanisms of epigenetics include, but are not limited to, DNA methylation (methylating of ciliary carbon at position 5), posttranslation modification of histone, chromatin remodeling (structural change), gene imprinting, RNA interference (noncoding RNA or gene silencing), etc. The epigenetic mechanisms of tumor cells have lost a fine regulation, and the breakdown of epigenetic patterns will lead to tumor phenotype expression. These mechanisms have been reviewed elsewhere, and here we will focus on DNA methylation in thyroid cancer.

DNA methylation is an important epigenetic change, which is persistent and hereditary. The methylation of promoter CpG can regulate gene expression and maintain chromosome integrity and DNA recombination. Based on 789 samples from the Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA) databases, the five-CpG signature could provide a novel biomarker with useful applications in thyroid cancer (PTC, FTC, ATC, and MTC) diagnosis and the diagnostic score formula on the condition of DNA methylation data [4]. However, the methylation pattern is unstable and can be reversed by small molecules and endogenous enzymes, leading to dedifferentiation and tumor heterogeneity. Abnormal DNA methylation, including a decrease in the overall methylation level of the genome, was accompanied by hypermethylation in some gene promoter regions. The hypermethylation of tumor suppressor gene promoter can reduce its expression, while the hypomethylation of tumor suppressor gene promoter can increase its expression, leading to tumorigenesis [5–8]. The following will describe the state of aberrant DNA methylation in different thyroid cancers.

2. DNA methylation in PTC

RET/PTC rearrangement and mutations in Ras and BRAF genes often occur in papillary thyroid carcinoma (PTC) [9]. In addition, many methylation of cancer suppressor genes are associated with BRAF gene mutations, such as Ras-association domain family 1A (RASSF1A), solute carrier family 5 member 8 gene (SLC5A8), retinoic acid receptor β2 (RARβ2), tissue inhibitor of metalloproteinase3 (TIMP3), phosphatase and tensin homolog deleted on chromosome ten (PTEN), metallothionein 1G (MT1G), ataxia-telangiectasia mutated (ATM), E-cadherin (ECAD), death-associated protein kinase (DAPK), multiple tumor suppressor 1 (MTS1 or P16), and mut-L homolog 1 (MLH1). Mutations of TSHR gene are not common in thyroid cancer, but high methylation and low expression of TSHR gene often occur. Recent studies have found that thyroid-specific genes (thyroid-stimulating hormone receptor and sodium/iodide symporter (TSHR and NIS), thyroid transcription factor-1 (TTF-1)) play an important role in occurrence and development of PTC. This part summarizes the related research on methylation genes in PTC in recent years (see **Table 1**).

2.1 DNA methylation of cancer suppressor genes in PTC

2.1.1 Ras association domain family 1 (RASSF1A)

RASSF1A is a member of Ras superfamily, which is located at chromosome 3 (exactly on 3p21.3). Hypermethylation of CpG islands in the RASSF1A promoter region contributes to epigenetic inactivation. It is a tumor suppressor gene widely

Sample sources	Main method	Related gene	Methylation (high/low)	Related gene expression (high/low)	BRAF mutation (+/−)	Cell pathway	Function	References
Tumor suppressor genes								
Patient tissue, normal people tissue	Western blotting analyses, RT-QPCR, fluorescent analysis	RASSF1A	High	Low	+	MAPK	Stabilize the microtubules	[12, 13]
		SLC5A8	High	Low	+		Sodium transporter	[17]
		RARβ2	High	Low	+		Negative regulation of cell cycle	[20]
		TIMP3	High	Low	+		Inhibitor of metalloproteinase	[23]
Patient tissue, normal people tissue	Methylation-specific PCR, RT-QPCR	PTEN	High	Low	+	PI3K/Akt	Inhibit PI3K/Akt pathway	[26]
		MT1G	High	Low	+		Metallothionein	[28]
		ATM	High	Low	+		Regulate cell cycle	[29]
Patient tissue, normal people tissue	Database query and retrospective medical chart, RT-PCR	ECAD	High	Low	+	Wnt/β-catenin	Mediate the adhesion of cells	[8]
Thyroid-specific genes								
Patient tissue, normal people tissue	Western blotting analyses, RT-QPCR	NIS	High	Low	+	TSHR/cAMP	Sodium transporter	[36]
		TSHR	High	Low	+		Thyrotropin receptor	[37]
		TTF-1	High	Low	+		Inhibit the pro-adipogenic response to pioglitazone	[40]
Other potential genes								
Patient tissue, thyroid cancer cell line	Western blotting analyses, RT-QPCR, bisulfite sequencing, and methylation-specific PCR	RASSF10	High	–	–	Others	Inhibit cell proliferation	[42]
		14-3-3σ	High	–	–		Cell cycle regulation	[45]
		RIZ1	High	–	–		Epigenetic mechanisms	[44]
		DACT2	High	–	–		Inhibiting Wnt signaling	[45]

Table 1.
Aberrant methylated genes in PTC.

expressed in various normal organs but is often deleted in tumors. It is speculated that BRAFV600E gene mutation in PTC regulated the RASSF1A-MST1-FoxO3 signaling pathway, which led to RASSF1A hypomethylation and affected the malignant degree of thyroid cancer. It is found that the methylation rate of RASSF1A in thyroid cancer is 15–75% [10]. Studies indicated RASSF1A methylation differed in PTC compared with normal thyroid and was correlated with extracapsular invasion inversely. It suggested that RASSF1A has a potential role as a molecular marker for characterization of PTC histopathology [11–15]. It is shown that hypermethylation of RASSF1A promoter region is 20–32% in PTC. Hypermethylation of RASSF1A in PTC was related to the multifocal and extracapsular invasion of tumors [16].

2.1.2 Solute carrier gene family 5A, member 8 (SLC5A8)

SLC5A8 is a passive iodine transporter located in the parietal membrane of thyroid follicular cells. SLC5A8 is not regulated by thyrotropin in normal thyroid tissues but methylated in thyroid tumors. Hypermethylation often occurred in the first exon of CpG islands in SLC5A8, which results in gene silencing and restoring expression inhibiting cancer cell growth. It has been pointed out that SLC5A8 was an anti-oncogene of colon cancer [17]. SLC5A8 was also frequently hypermethylated in thyroid cancer. Its function is unclear nowadays, but its hypermethylation might play a key role in the occurrence of thyroid cancer [18]. Studies revealed that SLC5A8 gene was highly methylated in typical PTC (90%) and only 20% in other types of PTC. In addition, low expression of SLC5A8 was also associated with BRAF T1796A, suggesting that SLC5A8 methylation may be important in MAPK pathway [19].

2.1.3 Retinoic acid receptor beta2

RARβ2 is a type of nuclear receptor that is activated by both all-trans retinoic acid and 9-cis retinoic acid, which has been shown to function as a tumor suppressor gene in different types of human tumors. It has been found that RARβ2 expression was decreased or deleted in tumors. It meant that RARβ2 inactivation was related to tumorigenesis. In the treatment of metastasis and recurrence of thyroid cancer, retinoic acid therapy could restore the iodine uptake ability of metastasis and then improved the efficacy of 131-I radiotherapy. Researches indicated that the methylation rate of RARβ2 in thyroid cancer was 14%, higher than that in normal thyroid tissues (7%). RARβ2 gene methylation was associated with BRAF gene mutation in Wnt/beta catenin pathway [20]. Studies found there was RARβ2 gene hypermethylation in thyroid cancer cell lines. And after treatment with 5-azacytidine, RARβ2 expression was significantly increased, and the growth of tumors was inhibited, while the inhibition still existed after removing 5-azacytidine. RARβ2 gene methylation took part in tumorigenesis and development in PTC [21].

2.1.4 Tissue inhibitor of metalloproteinases-3 (TIMP3)

TIMP3 can bind to matrix metalloproteinases (MMPs), inhibiting the activity of MMPs effectively [20, 22]. Methylation of TIMP3 promoter has been demonstrated in many malignant tumors. It is often associated with growth, invasion, and lymph node metastasis of malignant tumors. It is pointed out that BRAF mutation caused low expression of TIMP3 in PTC, which could cause invasion and progression of tumors. It was found that 38% of TIMP3 are hypermethylated in PTC [23].

2.1.5 Phosphatase and tensin homolog deleted on chromosome 10 (PTEN)

The PTEN gene is located on chromosome 10 (especially on the region of 10q23), which could encode a specific phosphatidylinositol triphosphate 3 dephosphorylation and inhibit the activation of PI3K/Akt signaling pathway. PTEN expression alteration is crucial to the pathogenesis of cancer and other diseases. Low level of PTEN caused by homozygous deletions, frameshift, nonsense mutations or hypermethylation, or PTEN protein destability occurs frequently in various human cancers [24, 25]. It was shown that PTEN gene expression was low in thyroid cancer. One study found the methylation status of PTEN in FA, FTC, and PTC. The results indicated that PTEN methylation level was gradually increased in PTC (45.7%), FA (83.3%), and FTC (85.7%). PTEN methylation was related to mutations genes in PI3K/Akt signaling pathway, such as PIK3CA and Ras genes, suggesting that PTEN methylation and PI3K/Akt signaling pathway played an important role in the process of occurrence and development in PTC [26].

2.1.6 Metallothionein 1G (MT1G)

MT1G, a member of the metallothionein family, is a highly conserved cysteine-rich small molecule, which is mainly involved in metal-related transport. MT1G exists in normal cells; it can regulate and maintain intracellular metal ion balance, cell proliferation, and apoptosis. MT1G promoter methylation is associated with decreasing gene expression, but not complete abrogation. Studies have shown that MT1G gene has abnormal methylation in thyroid cancer, liver cancer, colon cancer, and prostate cancer. It is confirmed that restoring MT1G gene expression could inhibit tumors growth in vivo and in vitro, suggesting that MT1G gene has anticancer effect [27]. It is shown that MT1G gene was abnormally methylated in thyroid cancer (30.3% in malignant tumors and 18.8% in benign tumors). Its expression was significantly decreased, and that methylation of MT1G gene was associated with its low expression. Further studies suggested that restoring MT1G gene expression could inhibit the growth and infiltration of PTC and induced cell cycle inhibition and apoptosis. The mechanism may inhibit PI3K/AKT pathway. In addition, hypermethylation of MT1G was also associated with lymph node metastasis [28].

2.1.7 Ataxia-telangiectasia mutated

ATM belongs to the PI3/PI4 enzyme family. Although there is emerging evidence for a role of ATM in promoting tumorigenesis, ATM signaling provides a barrier to activated oncogenes and tumor progression, rather than promoting cancer early in tumorigenesis. ATM is ubiquitous in human and other higher animal tissues and cells, such as testicular tissue. And a study showed that ATM was hypermethylated in PTC (50%) and 0% in normal thyroid tissue [29].

2.1.8 E-cadherin

ECAD is a family of transmembrane glycoproteins responsible for calcium-dependent cell adhesion. It is the key structural components of adherens junctions. It is reported that ECAD may also act as a gene transcriptional regulator. Two main mechanisms including hypermethylation of the promoter and microRNA imbalance have been widely studied under the ECAD regulation in head and neck tumors. The methylation of ECAD promoter region was accounted for 39.3% in PTC, and ECAD expression decreased in the early stage of tumorigenesis. The experiment data showed that methylation level of ECAD in thyroid cancer increased to 56% (18/32)

and 0% (0/27) in normal thyroid tissue. Further studies found that there was no significant correlation between ECAD methylation and T stage of lymph node metastasis in thyroid cancer. After 2.6 years of follow-up, the recurrence of thyroid cancer associated with ECAD methylation has no correlation [8].

2.1.9 Death-associated protein kinase

DAPK is a calmodulin-regulated ATK, which has an important role in the process of apoptosis. DAPK mechanism is largely due to promoter hypermethylation, leading to gene silencing. DAPK is ubiquitous in normal tissues. When the promoter of DAPK is methylated, it will cause an abnormal gene expression. Abnormal expression of DAPK can hinder the normal process of apoptosis and bring about tumorigenesis. And its low expression or deletion is one of the important mechanisms of cell carcinogenesis. It has been found that methylation of CpG island in the promoter region in DAPK is an important reason for expression silencing. The loss of DAPK protein in sporadic colorectal cancer is caused by the promoter hypermethylation. It existed in very small tumors. Therefore, the loss of DAPK gene plays an important role in the early stage of tumor formation. It is reported that DAPK promoter methylation accounts for 51% in PTC and abnormal methylation and DAPK gene silencing existed in many kinds of cancer cells [30]. It is pointed out that the high methylation level of DAPK gene was associated with tumor size and multiple lesions [31].

2.1.10 Multiple tumor suppressor 1 (MTS1 or P16)

MTS1 is hereafter called p16. It is an anti-oncogene in many tumors. The 5′-CpG fragment in the promoter region of P16 gene is the most susceptible to methylation, inhibiting its expression products. Abnormal expression of P16 gene can overactivate cyclin-dependent protein kinase 4 and stimulate abnormal cell proliferation, leading to tumorigenesis. Aberrant methylation of promoter region in P16 gene is the main cause for P16 gene inactivation [32]. Some scholars reported that the P16 gene was hypermethylated (35.9%) in PTC [33].

2.1.11 Mut-L homolog 1

MLH1 is one of the DNA mismatch repair genes located on the 3p21 region in chromosome 3. A correlation between MLH1 promoter methylation, specifically the 'C' region stops in MLH1 protein formation, can prevent the normal activation of DNA repair gene. Low expression of MLH1 gene was associated with BRAFV600E mutation and RET/PTC rearrangement. Hypermethylation of MLH1 promoter was found in colon cancer as well. A study indicated that abnormal methylation of MLH1 was significantly correlated with lymph node metastasis of PTC, suggesting that MLH1 might be a molecular marker of lymph node metastasis in PTC [34]. Another study found that there were abnormal methylation and low expression of MLH1 in thyroid cancer and MLH1 expression is associated with BRAF, IDH1, and NRAS gene mutations [35].

2.2 DNA methylation of thyroid-specific genes in PTC

2.2.1 Thyroid-stimulating hormone receptor and sodium/iodide symporter

On the other hand, methylation of thyroid-specific genes is also one of the causes for occurrence and development of PTC. Thyroid-specific genes mainly

include TSHR, NIS, thyroglobulin (Tg), and thyroid peroxidase (TPO), which participate in thyroid iodine uptake and maintenance of normal thyroid function. Under normal conditions, TSH stimulates TSHR on thyroid follicular epithelial cells and activates NIS to ingest iodine into cells. TSH is produced by the pituitary thyrotrophs and stimulates thyroid functions using TSHR. The iodine ingested synthesizes thyroid hormones catalyzed by TPO and is stored in Tg. It is found that abnormal expression of these molecules is related to iodine metabolism in PTC. Studies have shown that the expression of TSHR, NIS, Tg, and TPO with BRAF mutated in thyroid cancer is decreased [36]. In PTC, both TSHR and NIS are abnormally methylated, and their expression is decreased. Low expression of TSHR and NIS may be related to the occurrence and development of tumors. It also reduces the uptake of iodine capacity in tumor cells. Scholars found TSHR and NIS become an important cause for PTC in 131-I radiotherapy [37, 38]. In human and rabbit thyroid cancer cells, BRAFV600E mutant, a carcinogenic homolog of murine sarcomatous virulent bacterium, could cause activation of BRAF/MEK/MAPK signaling pathway and expression silencing of thyroid-specific genes including TPO, Tg, TSHR, and NIS. At last, it resulted in the reduction of iodine uptake in PTC.

2.2.2 Thyroid transcription factor-1

TTF-1 is known as thyroid-specific enhancer-binding protein (T/EBP). It is a transcription factor with homologous domains in the thyroid, lung, and central nervous system. TTF-1 gene is located in region 14q13.3 on chromosome 14. It is comprised of three exons and two introns. Under physiological conditions, TTF-1 is stable positive in thyroid tissue. TTF-1 can regulate the expression of thyroid-related genes such as TG, TPO, TSHR, and NIS. Thus it acts a pivotal part in regulating growth, development, and function of thyroid. It showed hNIS mRNA expression loss might be related to methylation of thyroid-specific transcription factor genes. Abnormal methylation caused loss of transcription factor expression with indirect loss of hNIS mRNA expression through the KAT-5 and KAT-10 responses to 5-azacytidine treatment with acquisition of parallel TTF-1 and hNIS mRNA expression. It was found that insufficient expression of TTF-1 and Pax-8 may result in the decrease of activity of thyroglobulin gene promoter in thyroid cancer cells. Some confirmed that TTF-1 gene was expressed lowly in thyroid cancer [39]. Other researchers studied the methylation status of TTF-1 in thyroid cancer and found that TTF-1 gene was highly methylated and lowly expressed in thyroid cancer cell lines, but not in normal thyroid [40].

2.3 DNA methylation of oncogene gene in PTC

At present, BRAF gene mutation has been found in melanoma, ovarian serous tumor, colorectal cancer, glioma, liver cancer, and leukemia. A large number of studies also showed that BRAF gene mutation in PTC is closely related to methylation of tumor suppressor. BRAF gene is an important transducer for Ras/Raf/MEK/ERK/MAPK pathways. About 90% of T1799A point mutation in BRAFV600E happen in PTC, which can increase BRAF activity. The BRAF gene plays its biological role by activating MEK/ERK signaling pathway. The result showed that PTC with BRAF gene mutation had strong tissue invasiveness and was easy to infiltrate tissues around thyroid gland [41]. Methylation of TIMP3 gene, SLC5A8 gene, and DAPK gene, which are tumor suppressor genes, are related to BRAF gene mutation and PTC invasion. The overexpression of BRAFV600E gene could lead to silencing of some thyroid-specific genes (like NIS, TG, TPO) in the activation state of

BRAF/MEK/MAPK pathway. It could cause iodine uptake activity decrease and ineffectiveness of radioiodine therapy. Therefore, it could affect thyroid cancer progression.

2.4 Other potentially aberrant methylation genes in PTC

In recent years, DNA methylation has become a new research hotspot. Many genes have been studied as potential abnormal methylation sites, including Ras association domain family 2 (RASSF2), Ras-association domain family 10 (RASSF10), disheveled-binding antagonist of beta-catenin 2 (DACT2), retinoblastoma protein-interacting zinc finger gene 1 (RIZ1), 14-3-3 protein family (14-3-3 sigma), and other signaling pathways such as JAK-STAT pathway, NF-kappa B pathway, HIF1 alpha pathway, and Notch pathway in thyroid cancer [42–45]. However, there are relatively few reports on these genes and pathways in thyroid cancer. Most of the mechanisms are not clear at present and need further study.

3. DNA methylation in FTC

DNA methylation analysis revealed 2130 and 19 differentially methylated CpGs in PTC and follicular thyroid carcinoma (FTC), respectively [46]. Aberrant DNA methylation of tumor suppressor genes is common in FTC and ATC. Certain specific tumor suppressor genes are mainly PTEN, RASSF1A, Rap1-GTPase activating protein, and thyroid-specific gene TRSH in FTC. This part summarizes the related research on methylation genes in FTC in recent years (see **Table 2**).

References	Gene	Function	DNA methylation	BRAFE	Incidence%
Tumor suppressor genes					
[39–42]	RASSFIA	RASSF1A localizes to microtubules and promotes their stabilization	↑	+	75% of FTC
[50]	RAP1GAP	RAP1GAPase-activating protein	↑	+	38% of FTC
[25]	PTEN	PTEN is involved in the regulation of cell cycle and preventing cells from growing and dividing rapidly	↑	+	85.7% of FTC
Thyroid-specific genes					
[58]	NIS	Sodium transporter	↑	+	53.8% of thyroid cancers
[58]	TSHR	Thyrotropin receptor	↑	+	47% of FTC
Oncogene genes					
[52, 53]	Maspin	A member of serine protease inhibitor	↑	+	100% of WDTC
Other genes					
[61]	DMCpG	—	↑	—	84% of FTC
[61]	RASAL1	—	↑	—	4.88% of FTC
[57]	COL4A2	—	↑	—	56% of FTC
[64]	RASSF10	—	↑	—	50% of FTC

Table 2.
Aberrant methylated genes in FTC.

3.1 DNA methylation of tumor suppressor genes in FTC

3.1.1 PTEN

PTEN negatively regulates AKT/PKB signaling pathway. It is involved in regulation of cell cycle, cell growth inhibition, and rapid division [47]. Aberrant DNA methylation in this gene is also mostly reported in FTC. It confirmed that PTEN promoter hypermethylation was detected in six of seven (85%) FTC and five of six (83.3%) follicular adenomas. The results showed a high frequency of PTEN promoter hypermethylation, especially in follicular tumors. It means that it has a possible role in thyroid cancer [25]. Studies found methylation status of PTEN in FA and FTC. And PTEN methylation level was gradually increased in FA (83.3%) and FTC (85.7%). Authors have shown that methylation of PTEN promoter plays an important role in FTC [48].

3.1.2 RASSF1A

Differential expression of RASSF1A gene is related to occurrence of thyroid cancer. Aberrant DNA methylation is an important mechanism of RASSF1A gene inactivation. Studies found 44% benign adenomas, 75% follicular thyroid cancers tumors, and 20% PTC tumors harbored promoter methylation in greater than or equal to 25% of RASSF1A alleles by real-time quantitative methylation-specific PCR [39–42]. Methylation frequency was higher in invasive thyroid cancer. It was found to be 70% of the RASSF1A methylation rate in FTC, 80% in MTC, and 78% in UTC compared with benign PTC [14]. Inactivation of RASSF1A in different stages of thyroid cancer was detected by tumor metastasis classification, and compared with FTC, only a small part of RASSF1A methylation in PTC is abnormal. These studies indicated follicular cell-derived thyroid tumorigenesis may be an early step [15, 49].

3.1.3 Rap1-GTPase-activating protein

RAP1GAP gene encodes a type of GTPase-activating protein that downregulates Ras-related protein activity. Ras oncoproteins are very important for both development and maintenance of many tumor types. RAP1GAP is involved in the regulation of mitosis and carcinogenesis in thyroid cells. Researchers aimed to determine the global patterns of aberrant DNA methylation in thyroid cancer using DNA methylation arrays [50]. And the study identified 262 and 352 hypermethylated and 13 and 21 hypomethylated genes in PTC and FTC, respectively. In addition, 86 and 131 hypermethylated genes were identified. Among these genes, four potential oncogenes (INSL4, DPPA2, TCL1B, and NOTCH4) were frequently regulated by aberrant methylation in primary thyroid tumors [51].

3.1.4 Mammary serine protease inhibitor (Maspin)

Besides, a member of the serine protease inhibitor superfamily named Maspin is a unique tumor suppressor gene encoding SERPINB5 gene. Epigenetic changes of Maspin expression occurred in the 5′ regulatory region of Maspin gene and involved cytosine methylation, histone deacetylation, and chromatin accessibility. The epigenetic deregulation frequently participates in tumorigenesis by inactivation of tumor suppressor genes. The association of promoter hypermethylation and gene silencing is an established oncogenic process in cancer. Promoter methylation of Maspin gene could lead to gene silencing in thyroid cancer, breast cancer, skin cancer, and colon cancer. Studies have indicated that overexpression of Maspin in

gastric cancer, pancreatic cancer, and ovarian cancers resulted from CpG promoter of Maspin demethylation. A study detected DNA methylation status in Maspin promoter region, indicating that overexpression of the gene was the result of DNA hypomethylation [52]. It was closely related to the morphological dedifferentiation of thyroid cancer. Another study found 100% Maspin hypermethylation was closely associated with morphological dedifferentiation in thyroid cancers [52, 53].

3.2 DNA methylation of thyroid-specific genes in FTC

In addition to tumor suppressor genes and oncogenes, hypermethylation could lead to NIS and TSHR gene silencing in FTC, too. NIS methylation is of great significance in treatment of thyroid cancer. Therefore, abnormal methylation of these genes may be the pathogenesis or progression factor in FTC [54, 55]. Aberrant promoter methylation was examined in 24 tumor suppressor genes using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay and methylation-specific PCR (MSP). In thyroid cancers, CASP8 (caspase-8), RASSF1, and NIS were methylated in 9/13, 10/13, and 7/13, respectively [56]. Some researches also found combination gene panels TPO and UCHL1 (ROC = 0.607, sensitivity 78%) discriminated FTC from FA and RASSF1 and TPO (ROC = 0.881, sensitivity 78%) discriminated FTC from normal. Methylation of TSHR distinguished PTC from FTC (ROC = 0.701, sensitivity 84%) and PTC from FA (ROC = 0.685, sensitivity 70%) [57]. And the six-gene panel of TIMP3, RARβ2, SERPINB5, RASSF1, TPO, and TSHR, which differentiates PTC from normal thyroid, had the best combination sensitivity (91%) and specificity (81%) of the panels addressing discrimination of cancer tissue by quantitative methylation-specific polymerase chain reaction (QMSP) in a retrospective cohort of 329 patients [58, 59].

3.3 Other abnormal methylation genes in FTC

Nowadays, there are a few studies on methylation of FTC genes. However, many genes still have been studied as potential abnormal methylation sites in FTC. For example, one of the ZIC families called ZIC1 (C2H2-type zinc finger proteins) is frequently hypermethylated in FTCs [60]. Moreover, 3564 differentially methylated CpGs (DMCpG) were detected in FTC and 84% hypermethylated with respect to normal controls. It is suggested that perturbed DNA methylation, in particular hypermethylation, is a component of the molecular mechanisms leading to FTC formation and that DNA methylation profiling might help in differentiating FTCs from their benign counterpart [61]. Also, others identified the presence of RASAL1 mutations, with a prevalence of 4.88% (n = 2 of 41) in FTC and 16.67% (n = 5 of 30) in ATC [62]. Studies found a more detailed analysis showing that 53.9% of the hypermethylated and 81.5% of the hypomethylated CpG sites identified in differentiated primary tumors (PTCs and FTCs) were also present in differentiated thyroid carcinoma-derived cancer cell [61]. Aside from that, COL4A2 was hypermethylated in 56% of the FTC samples by array measurement in the discovery series [57, 63]. Another study indicated that RASSF10 was frequently hypermethylated in thyroid cancer. It showed 50% of methylation frequency of RASSF10 in FTC and the highest (100%) in MTCs [64].

4. DNA methylation in ATC

Promoter methylation of PTEN is also common in anaplastic thyroid cancer (ATC) [65]. PTEN methylation is related to gene changes of PI3K Akt pathway in

thyroid tumors, including PTEN mutations, various subtypes of Ras mutations, PIK3CA mutations, and amplification [66, 67]. One study analyzed 24 genetic alterations in the major genes of MAPK and PI3K-AKT pathways in 48 ATC samples and found that the majority of (81%) samples that harbored genetic alterations could be likely activated in both pathways [68]. Accordingly, another DNA methylation pan-cancer study focused on promoters found that thyroid carcinoma exhibited one of the lowest frequencies in both hypomethylation and hypermethylation events. And ATC exhibits a high frequency of DNA methylation alterations (tenfold higher than PTC) [69, 70]. 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, 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 [71, 72].

In addition, solute carrier family 26, member 4 (SLC26A4) gene, encodes a transmembrane protein named pendrin with up to 15 predicted membrane spanning domains and affects the flow of iodine into follicular lumen. The following were reported: 71% of ATC, 44% of benign tumors, 46% of FTC, and 71% of PTC, with abnormal SLC26A4 gene methylation in 64 cases of primary thyroid tumors and 6 cases of thyroid tumor cell lines [18, 73]. In addition, 81.5% of hypermethylated genes and 89% of hypomethylated genes were also present in nondifferentiated primary tumors (MTCs and ATCs) and nondifferentiated thyroid carcinoma-derived cancer cell lines [74], while Ras protein activator like-1 gene (RASAL1) displayed MAPK- and PI3K-suppressing and thyroid tumor-suppressing activities, which can be impaired by the mutations. Hypermethylation and mutations of RASAL1 were found in 33.33% (n = 10 of 30) of ATCs and in 0 of 20 (0%) of benign thyroid tumors [62]. However, ATC showed more hypomethylation than hypermethylation events, indicating that hypomethylation is related to dedifferentiation [70]. The authors validated four genes (NOTCH4 and TCL1B in ATCs, INSL4 and DPPA2 in MTCs) that become aberrantly hypomethylated in nondifferentiated thyroid tumors. All of them have been proposed to have an oncogenic role in cancer. And NOTCH4 (a member of the Notch family of transmembrane receptors) is frequently overexpressed in thyroid tumors [75, 76].

This part summarizes the related research on methylation genes in ATC in recent years (see **Table 3**).

Authors	Gene	Function	DNA methylation	BRAFE	Incidence%
Oncogene genes					
[70]	TCL1B	An oncogene frequently activated by reciprocal translocations	↑	+	64% of ATC
[70]	NOTCH4 A	Member of notch family, which plays a role in a variety of developmental processes	↑	+	45% of ATC
Thyroid-specific genes					
[18, 73]	SLC26A4	Dysfunctional pendrin	↑	—	71% ATC
Tumor suppressor genes					
[25]	PTEN	PI3K–AKT pathway	↑	—	81% of ATC
[62]	RASAL1	MAPK- and PI3K-suppressing	↑	—	33.33% of ATC

Table 3.
Aberrant methylated genes in ATC.

5. DNA methylation in MTC

Medullary thyroid cancer is a neuroendocrine tumor originating from parafollicular C cells, and it is highly resistant to chemo- and radiotherapy [77]. Spry1 is a candidate tumor-suppressor gene in MTC. The hyper-activation of PI3K/Akt/mTOR cascade has a relevant role in the pathogenesis and progression of MTC. In fact, most of pro-oncogenic effects of RET and Ras mutations are modulated by the activation of PI3K/Akt/mTOR pathway [78, 79]. For patients with metastatic disease, standard treatment modalities include local treatments (radiofrequency ablation, radiation therapy, embolization) and systemic treatment chemotherapy and more recently tyrosine kinase inhibitors (TKIs) targeting RET protein. As experience has been limited to case reports or case series, response rates (RR) ranged from 0 to 25% for periods of up to a few months. In nondifferentiated thyroid tumors, INSL4 and DPPA2 become aberrantly hypomethylated, both of which have been proposed to have an oncogenic role in MTC.

5.1 Sprouty1 (Spry1)

Sprouty (Spry) family of genes is composed of four members in mammals (Spry1–4). The Spry1 promoter is frequently methylated in MTC, and that Spry1 expression is consequently decreased. These findings identify Spry1 as a candidate tumor-suppressor gene in MTC. In mammals, the situation is more complicated as Spry proteins have been shown to be activators or inhibitors of receptor tyrosine kinase signaling depending on the cellular context or the receptor tyrosine kinase analyzed [80, 81]. Spry family members have been proposed to function as tumor-suppressor genes in a growing list of cancerous malignancies, including prostate and hepatocellular carcinoma, B-cell lymphoma, or neuroblastoma [82]. Finally, the authors found that SPRY1 promoter is frequently methylated and its expression decreased in human MTC. The mRNA levels of Spry1 are detected in murine C cells of thyroid with real-time RT-PCR. In situ hybridization showed expression of Spry1 mRNA in the fourth pharyngeal pouch, where thyroid C-cells originate and beta-galactosidase staining of thyroids from 1-month-old Spry1LacZ/þ mice. One study used the hypomethylating agent 5′-aza-deoxycytidine (5′-Aza-dC) to confirm a causal relationship between promoter methylation and Spry1 expression [83]. As expected, TT cells treated with 5′-aza-dC showed an increase of approximately sixfold in the levels of Spry1 mRNA when compared to vehicle-treated cells. 5′-Aza-deoxycytidine (AZA), a demethylating agent, is in combination with the mTOR inhibitor everolimus in MTC cells (MZ-CRC-1 and TT). An innovative bioinformatic pipeline identified four potential molecular pathways implicated in the synergy between AZA and everolimus: PI3K-Akt signaling, the neurotrophin pathway, ECM-receptor interaction, and focal adhesion. Among these, the neurotrophin signaling pathway was most directly involved in apoptosis, through NGFR and Bax gene overexpression. Increased expression of genes involved in the NGFR-MAPK10-TP53-Bax/Bcl2 pathway during incubation with AZA plus everolimus was validated by western blotting in MZ-CRC-1 cells [84].

5.2 Insulin-like 4 (INSL4)

INSL4 (pro-EPIL) belongs to the insulin and insulin-like growth factor family and is expressed strongly during the first trimester of pregnancy by the differentiated syncytiotrophoblast [85]. It has been shown to be overexpressed in breast tumors with an aggressive phenotype [86], but the underlying mechanisms are still

unknown. The aberrant overexpression of INSL4 in breast tumors, together with the aberrant promoter hypomethylation reported in this study, suggests that promoter demethylation might be a frequent mechanism of activation of INSL4 oncogene activation in cancer.

5.3 Developmental pluripotency-associated 2 (DPPA2)

DPPA2 is expressed early in the embryo's development [87] but also in some tumor types [88]. Although the underlying molecular mechanism has not been reported yet, the authors' data indicated that promoter hypomethylation might play an important role. The frequent promoter hypomethylation observed in nondifferentiated tumors might be relevant for treatment with demethylating drugs [51, 88].

6. Conclusion

More and more researches have realized that the occurrence of tumors is not only entirely determined by genes but also epigenetics. The changes of epigenetics in thyroid cancer are mainly manifested in the aberrant methylation of tumor suppressor genes and thyroid-related genes. Numerous studies on DNA methylation in thyroid cancer have improved our understanding of thyroid carcinogenesis. Some of the recent findings, including 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 most likely to lead the direction of future research in the field of DNA methylation in thyroid cancer. A large number of studies confirmed the importance of DNA methylation as a source of novel biomarkers for early diagnosis, therapeutic perspective, and prognosis evaluation in thyroid cancer. In addition, the design of specific target demethylation drugs, which reactivate the function of tumor suppressor genes, is expected to become a new scheme for cancer treatment. Therefore, further functional experiments in vitro and in vivo are necessary for better understanding of the meaning and potential mechanism of DNA methylation changes in thyroid cancer as well as the evaluation of candidate biomarkers through case-control studies and prospective trials.

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