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Epigenetic Events Suggest A Distinct Molecular Pathogenesis In Braf-Associated Papillary Thyroid Cancer

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EPIGENETIC EVENTS SUGGEST A DISTINCT MOLECULAR PATHOGENESIS IN BRAF-ASSOCIATED PAPILLARY THYROID CANCER

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EPIGENETIC EVENTS SUGGEST A DISTINCT MOLECULAR PATHOGENESIS IN BRAF-ASSOCIATED PAPILLARY THYROID CANCER.

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Papillary thyroid cancer (PTC) associated with a somatic V-raf murine sarcoma viral oncogene homolog B1 (BRAF) V600E mutation has been associated with a more aggressive phenotype (i.e. extrathyroidal extension, lymph node metastasis, high TNM stage and recurrence) than those with wildtype (WT) BRAF. The underlying molecular mechanism for this association is incompletely clarified. Epigenetic alterations, such as DNA methylation participate with genetic abnormalities to cause altered patterns of gene expression and/or function leading to oncogenesis. Using a quantitative, genome-wide approach to evaluate the PTC DNA methylome, we identified a distinct DNA methylation profile in BRAF-associated PTC. This represents the first, unbiased, systematic, quantitative genome-wide evaluation of DNA methylation alterations in PTC.

A distinct DNA methylation profile was noted in BRAF V600E PTC versus BRAF WT PTC. Twenty-four genes were significantly hypermethylated in BRAF V600E PTC versus BRAF WT ($p<0.005$ for all). Genes frequently and significantly hypermethylated in BRAF V600E PTC included those involved in transcriptional regulation and cell cycle control such as CDKN2B/p15, RASSF1A and CD6. 25 BRAF V600E PTC and 25 BRAF WT PTC were used for validation of the methylation profile of RASSF1 and analysis of its gene expression. One of two CpG islands in the RASSF1A promoter was differentially methylated with an average of 73% and 8% methylation in BRAF V600E PTC and

BRAF WT PTC respectively. In addition, 92% (n=23) of the BRAF V600E tumors were hypermethylated at this CpG island, compared to 12% (n=3) of the BRAF WT tumors. Expression of RASSF1A was decreased in BRAF V600E PTC relative to expression in BRAF WT PTC.

The DNA methylation profile of PTC correlates with BRAF mutational status, underscoring its importance in PTC development. The molecular mechanism of the more aggressive phenotype of BRAF V600E PTC may be related to aberrant DNA methylation of genes involved in transcriptional and cell cycle control. Functional studies assessing the effect of azanucleoside, a demethylating agent on RASSF1A gene expression may further determine a cause-effect relationship. Furthermore, conducting such functional experiments in BRAF V600E PTC and BRAF WT PTC cell cultures would be critical in assessing the possibility of clinically applying a demethylating agent to treatment of aggressive, BRAF V600E PTC.

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INTRODUCTION

Thyroid carcinoma is the most common endocrine malignancy. Papillary thyroid cancer (PTC) accounts for over 90% of thyroid malignancies[1]. With improved diagnostic techniques, papillary thyroid carcinoma is identified much more frequently than in the past[2]. Currently, the majority of patients present with a thyroid nodule and are often asymptomatic. Symptomatic patients, however, may present with concomitant vocal cord dysfunction or subglottic/tracheal mass, recurrent disease in the central compartment, symptomatic disease with voice changes, dyspnea, hemoptysis or dysphagia, and finally, patients with documented invasive disease based on pre-operative imaging[3]. More papillary thyroid cancers are diagnosed as microcarcinomas, therefore molecular methods of detecting aggressive disease will aid in treatment planning.

Genetic Alterations in Papillary Thyroid Cancer

The majority of genetic alterations in thyroid cancer exert their oncogenic actions at least partially through the activation of the RAS/mitogen-activated protein kinase/Extracellular signal-regulated protein kinase (MAPK/ERK) pathway. Constitutive activation of the MAPK/ERK pathway leads to tumorigenesis by upregulating cell division and proliferation[4]. Activation of this pathway is a common and important mechanism in the genesis and progression of human cancers. When constitutively activated, the MAPK/ERK pathway leads to tumorigenesis[4]. Alterations of RET/PTC, BRAF and RAS genes are linked to papillary thyroid tumorigenesis.

The RAS/mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated protein kinase (ERK) pathway

The mitogen-activated protein kinases are proline-targeted serine/threonine kinases involved in the RAS/mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated protein kinase (ERK) signal transduction pathways that regulate nuclear gene transcription in response to changes in the cellular environment (Turjanski, 2007).

The MAPK/ERK pathway is activated by a GTP-bound active RAS protein, which is activated by binding of an extracellular growth factor to a growth factor receptor in the cell membrane causing receptor phosphorylation and interaction with a nucleotide exchange factor, SOS, and inactive GDP-bound RAS. Activation of RAS results in a conformational change that allows it to interact with RAF, a serine-threonine kinase and bring it to the plasma membrane. RAF phosphorylates and activates MAPK/ERK kinase, which in turn phosphorylates ERK kinase. Phosphorylated ERK translocates to the nucleus, targeting transcription factors.

Activation of the RAS/MAPK/ERK kinase signal transduction pathway is involved in transcription of nuclear oncogenes such as MYC and FOS, and cell cycle components such as cyclin D1. This pathway controls cellular functions involved in tumorigenesis, including cell proliferation, differentiation, migration and apoptosis [5-7].

Figure 1. The mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated protein kinase (ERK) pathway

RET/PTC

RET proto-oncogene encodes a cell membrane receptor tyrosine kinase. Ligands of the kinase are of the glial-cell line-derived neurotrophic factor (GDNF) family which cause the receptors to dimerize upon binding, leading to auto-phosphorylation of tyrosine residues and initiation of the MAPK/ERK pathway signaling cascade[8]. High expression of RET in parafollicular C-cells of the thyroid gland are consistent with its role in development of neural crest-derived cell lineages. This high expression of RET does not occur in thyroid follicular cells, however, RET activation in these cells occurs by fusion of the 3' tyrosine domain of RET to the 5' portion of constitutively expressed genes.

The most common RET/PTC rearrangements seen in PTC are RET/PTC1 (fusion with H4 gene) and RET/PTC3 (fusion with NCOA4 gene). Prevalence of RET/PTC rearrangements in PTC is greatest in populations exposed to radiation (60–70%). In the general population, the prevalence is higher in children[6]. Activation of RET/PTC results in downregulation of thyroglobulin and sodium iodide importer genes, which are thyroid specific, and cell differentiation. Wildtype RET/PTC and truncated forms of RET/PTC activate a number of other pathways, including the PI3K/AKT pathway, contributing to its biological activity[9].

BRAF

Mutations of BRAF, a serine-threonine kinase and downstream signaling molecule of RAS and RET, are potent activators of the MAPK/ERK pathway[4,10]. These missense mutations of the BRAF gene, located on chromosome 7, occur in the kinase domain with the T1799A transversion mutation resulting in a single amino acid

substitution of valine to glutamic acid (V600E) accounting for 80-90% of BRAF activating mutations[6,11]. The V600E mutation is thought to mimic phosphorylation in the activation segment of BRAF by inserting a negatively charged residue adjacent to an activating phosphorylation site[4].

BRAF V600E occurs as a sporadic mutation in thyroid cancer[11,12] and is restricted to papillary and anaplastic or poorly differentiated carcinomas[13]. Prevalence of the mutation is reported in papillary thyroid cancer at 35-40%, with a significantly higher prevalence in males than females[11]. The rate of BRAF mutation in PTC is the second highest to that in melanomas (60%) and is much higher than other cancers such as colorectal adenocarcinomas (5–10%) and lung cancers (1.8%)[11]. BRAF V600E correlates with poorer clinicopathologic outcomes defined as extrathyroidal extension, lymph node metastasis, and advanced tumor grade (III/IV) at presentation, and is prognostic of tumor recurrence[14,15].

Although not confirmed by similar studies[12], 38% of papillary thyroid tumors showed RET/PTC rearrangement[11], contrary to reports that BRAF V600E mutation is does not occur with RET/PTC or RAS mutations in cancer[12]. The low oncogenic potential of both BRAF and RET/PTC1 suggest that both mutations occurring in the same pathway are not necessarily redundant, but may cooperate in papillary thyroid tumorigenesis. Indeed, RET/PTC1 and RAS mutations have been shown to synergistically lead to tumorigenesis[11].

RAS

Activating mutations of the three RAS oncogenes (H-RAS, K-RAS, and N-RAS) occur in thyroid tumors, however, their prevalence depending on histology of the tumors has been controversial[16]. Early studies demonstrated that RAS mutations were more frequent in follicular tumors than papillary thyroid cancers, in addition to different patterns of mutations occurring in the two types. Indeed, mutations in up to 50% on microfollicular adenoma further supported the idea that RAS oncogene activation was an early event in follicular thyroid tumorigenesis. More recent studies have reported varying incidences of RAS mutations in thyroid tumors (0-50% in PTC, 0-85% in adenomas, 14-62% in FTC, and 0-60% in anaplastic carcinomas). Some investigators find no correlation between RAS mutation isoforms and tumor pathology, while others report a higher frequency of mutations in codon61 of H-RAS and N-RAS in FTC and poorly differentiated carcinomas.

The molecular pathogenesis of PTC thus remains incompletely clarified. With respect to gene alterations, papillary thyroid cancers have relatively low rates of loss of heterozygosity, with no specific region displaying a particularly high prevalence when compared to follicular thyroid cancers[17]. Like RET, BRAF or RAS mutations, other molecular alterations are thought to be essential for the induction of papillary thyroid cancer. Epigenetic events are very likely to contribute to significant variation in gene expression profiling, phenotypical features, and biologic characteristics seen among papillary thyroid carcinoma[18].

Familial Nonmedullary Thyroid Cancer

Although mostly sporadic, there is some evidence for a familial form of nonmedullary thyroid cancer (NMTC), which includes PTC, not associated with known Mendelian syndromes. Familial nonmedullary thyroid cancer (FNMTC) is thought to cause a more aggressive disease marked by younger age at presentation, multifocality, local invasion, lymph node metastasis and tumor recurrence. Linkage analysis data is limited by the small size and inconsistent inclusion criteria of the studies performed, but point to a polygenic mode of inheritance in FNMTC. Perhaps the most important result of these studies is the exclusion of several genes, namely the adenomatous polyposis coli (APC), phosphate and tensin homolog (PTEN), thyroid-stimulating hormone receptor, rearranged during transfection (RET), tropomyosin receptor kinase, hepatocyte growth factor receptor (MET), TRKA, JUNB, and BRAF genes.

Epigenetics mechanisms involved in tumorigenesis

Epigenetic silencing of regulatory genes is part of the global genomic alterations in cancer that alter pathways relevant to stem cell growth and differentiation. Epigenetic silencing mechanisms include covalent modifications of chromatin, DNA cytosine methylation, non-coding RNAs, and nucleosome remodelling[19]. It has been proposed that epigenetic abnormalities may play a seminal role in the earliest steps in tumorigenesis[20-23]. Epigenetic changes may act in concert with genetic changes resulting in tumorigenesis, because they are mitotically heritable. The high degree of mitotic stability of silencing plus the progressive nature by which it is achieved makes pathological silencing of growth controlling, and other genes essential to carcinogenesis.

Patterns of DNA methylation are linked to gene expression; for example, methylation in a gene promoter region generally correlates with a silenced gene[24]. DNA methylation, the DNA methyltransferase (DNMT) catalyzed addition of methyl group to cytosine ring is restricted to cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide) in humans and other mammals[24]. The distribution of CpG dinucleotides in the genome is unusually asymmetric, occurring in small clusters called "CpG islands". The CpG islands are often in promoter regions of genes and are usually unmethylated regardless of the transcriptional state. This highlights the importance of DNA methylation for gene expression, especially in transcriptional silencing[24].

Aberrant DNA methylation plays a strong role in tumorigenesis. Global hypomethylation of intergenic CpG dinucleotides and regional hypermethylation of CpG islands in promoter regions are characteristic hallmarks of many cancers[23]. The impact of hypermethylation on tumorigenesis is further illustrated by the silencing of multiple tumor suppressor genes, thereby contributing to the hallmarks of carcinogenesis which include evading apoptosis (P53, p14ARF, BNIP3, Caspase-8), insensitivity to antigrowth signals (p16INK4a, miR-124a), sustained angiogenesis (TIMP3, TSP1), limitless replicative potential (hTERT), and tissue invasion and metastasis (E-cadherin, LIMS2).

Transcriptional silencing is also a result of chromatin compaction due to convergence of DNA methylation and histone modifications. Methylated DNA recruits methyl-binding proteins (MBDPs), which have methyl-CpG-binding domains (MBD), to hypermethylated DNA. MBDPS also associate with histone deacetylases, resulting in chromatin remodeling and gene silencing. In addition to these mechanisms of silencing,

histone methyltransferase (HMTs) repress transcription by methylation of lysine 9 of histone 3 (H3K9) or lysine 27 of histone 3 (H3K27).

The influence of epigenetic events on tumorigenesis is well illustrated the evolution of colon cancer, in which risk factors for common cancers such as aging and inflammation are shown to cause expansions in either normal colon epithelial stem cells or precursor cells derived from them. Epigenetic gatekeepers such as cyclin-dependent kinase inhibitor 2A (CDKN2A/p16), secreted frizzled-related protein (SFRP), GATA-binding protein 4 and 5 (GATA-4 and -5), and adenomatous polyposis of the colon (APC) prevent early tumor progression in colon cancer. Normal epigenetic modulation of these gatekeeper genes allows them to prevent stem/precursor cells from becoming immortalized during periods of chronic stresses and renewal pressures on cell systems. APC is a classically mutated tumor suppressor gene in colon cancer, which is also inactivated by epigenetic mechanisms[25]. Epigenetic silencing of one allele serves as a second-hit in Knudson's hypothesis for tumor suppressor gene inactivation when paired which mutations on the other allele[20].

Like APC, loss of p16 can be epigenetically mediated, permitting expanding cells to develop genomic instability[26,27] and further epigenetic gene-silencing events[28]. Its loss is seen in subsets of pre-invasive stages of colon and other cancers[28]. Finally, GATA-4 and -5 transcription factor genes important for both embryonic gastrointestinal epithelial development and for maturation in adults are epigenetically silenced in about half of all the pre-invasive and invasive lesions for colon cancer[29]. This can hamper differentiation and promote precursor cell expansion.

The wingless-type MMTV integration site (Wnt) pathway activation also illustrates how multiple epigenetic events may act in concert to affect a single-cell pathway. Inappropriate silencing of these genes leads to abnormal activation of the Wnt pathway, which plays a canonical role in colon tumorigenesis[22]. These genes are independently affected by epigenetic events but result in Wnt activation. Four genes in the SFRP family encode proteins that antagonize the action of the Wnt ligand at the cell membrane are hypermethylated simultaneously in the majority of pre-invasive lesions for colon cancer. Upregulation of the survival protein Sirtuin 1 (SIRT1) also results in Wnt pathway activation. SIRT1 is upregulated as a result of loss of the transcription factor hypermethylated in cancer 1 (HIC1) via hypermethylation in early pre-invasive lesions in colon cancer as well as other types of cancer[22]. Loss of HIC1 also results in additional gene silencing events as well as downregulation of tumor protein 53 (p53).

Loss of DNA methylation results in weakening of transcriptional repression in normally silent regions of the genome resulting in harmful expression of inserted or normally silenced genes, and loss of functional stability of chromosomes. It has been established that covalent histone modification is linked to DNA methylation. Cytosine methylation attracts methylated DNA-binding proteins and histone deacetylases to methylated CpG islands during chromatin compaction and gene silencing[30,31]. In addition to epigenetic modification of transcriptional start sites, there is evidence for more global changes in chromatin structure. For instance, there is an overall decrease in the 5-methylcytosine content of cancer genomes that is reflected as hypermethylation in CpG islands[32]. The consistently observed hypermethylation is due to a change in 5-methylcytosine distributions rather than an overall increase in total amount of

methylation. It has also been observed that large stretches of DNA can become abnormally methylated in cancer.

Epigenetics of papillary thyroid cancer

Quantitative analysis of promoter hypermethylation in thyroid cancer has involved RASSF1A, TSHR, RARβ2, DAPK, S100, p16, CDH1, CALCA, TIMP3, TGF-β, and GSTpi[33]. Hypermethylation of 2 or more markers (RASSF1A, TSHR, RARβ2, DAPK, CDH1, TIMP3, TGF-β) was detectable in 25% of thyroid hyperplasias, 38% of adenomas, 48% of thyroid cancers, and 100% of cell lines. Rank correlation analysis of marker hypermethylation suggests that a subset of the markers were hypermethylated in concert, which may represent a thyroid-specific regulatory process[33]. Additionally, a positive correlation was observed between BRAF mutation and RARβ2, and a negative correlation between BRAF mutation and RASSF1A[33].

Investigation of DNA methylation in PTC has been predominantly restricted to individual candidate tumor suppressor genes and genes known for their role in thyroid function, using locus specific non-quantitative methods. BRAF, RASSFIA, TSHR, ECAD, NIS-L, ATM, DAPK, SLC5A8, TIMP3, and RARβ2 have been analyzed for DNA methylation. Promoter hypermethylation of TSHR, NIS-L, ATM, and ECAD has been demonstrated in 34-59%[34,35], 22%, 50%, and 56% of patients with papillary thyroid cancer respectively[35].

Thyroid-stimulating hormone receptor (TSHR) and sodium iodide symporter (NIS)

TSHR stimulates several key steps in thyrocyte concentration of iodine, including uptake by NIS and oxidation before incorporation into thyroglobulin by thyroid peroxidase[34]. The methylation status of the NIS and TSHR promoter regions are important because these genes are specific to the thyroid may play a role in the uptake of iodine and normal cellular function[35]. Promoter hypermethylation resulting in decreased expression of TSHR and NIS may result in a decreased ability to concentrate iodine, rendering ablative doses of ^{131}I ineffective[35]. Promoter hypermethylation of TSHR is reported in 34-59%[34,35] of patients with papillary thyroid cancer. NIS mRNA expression has been shown to be decreased in thyroid cancers[35,36], and this has been proposed to be secondary to methylation of the promoter region[35,32]. The NIS-L region within the promoter was shown to be hypermethylated in 22% (7/32) of patients with papillary thyroid cancer[35], but was not methylated in surrounding histologically benign tissue.

E-cadherin (ECAD)

E-cadherin complexes with catenins to promote Ca2+-dependent, homotypic cell-to-cell adhesion and to establish normal epithelial tissue architecture[37]. Disruption of the E-cadherin/catenin complex contributes to tumor metastasis, and decreased expression of E-cadherin is observed in advanced stage, poorly differentiated carcinomas[37]. Promoter hypermethylation has been demonstrated in multiple human cancers, including papillary thyroid cancer in 56% (18/32) of patients[35].

Ataxia telangiectasia mutated (ATM)

ATM is a member of the phosphatidylinositol 3-kinase family of proteins that respond to DNA damage by phosphorylating key substrates (p53, BRCA1) involved in DNA repair and/or cell cycle control[38-40]. Hypermethylation of ATM promoter was observed in 50% (16/32) of patients with papillary thyroid cancer analyzed[35]

Apical iodide transporter (AIT)

The thyroid apical iodide transporter AIT encoded by the SCL5A8 gene has been defined as a sodium-coupled transporter of short-chain fatty acid. It is thought that AIT may be involved in the passive transport of iodide from thyrocyte to the follicle lumen[41,42]. Expression of SCL5A8 is decreased in thyroid cancers compared to other iodide transporters[41], and is expressed abundantly in colon cancer, functioning as a tumor suppressor gene. Silencing of SLC5A8 occurs by promoter hypermethylation in about 50% of colon cancer cell lines and primary colon cancers. Decreased expression of SLC5A8 observed in classical variant of papillary thyroid cancer is linked to hypermethylation of exon 1 of the gene[42].

Hypermethylation occurred in 33% (76/231) of PTC and was associated with extrathyroidal invasion (40%) and multifocality (40%)[43]. This epigenetic event is thought occur at a later stage in papillary thyroid cancer and specific of the classical variant, therefore may be secondary to other genetic alterations occurring selectively in the tumor type[42]. Indeed, SLC5A8 and BRAF discriminate the classical variant PTC, supporting the argument. In addition, a strong association between low SLC5A8 expression and the

presence of BRAF V600E[42] or advanced clinicopathologic features[43] suggests a link in the progression to more aggressive papillary thyroid cancer.

The tissue inhibitor of metalloproteinase 3 (TIMP3)

TIMP3 is one of 4 tissue inhibitors of metalloproteinase thought to inhibit growth, angiogenesis, invasion, and metastasis in several human cancers[43,44]. TIMP3 inhibits vascular endothelial factor (VEGF)-mediated angiogenesis by blocking the binding of VEGF to VEGF receptor-2, thereby inhibiting downstream signaling and angiogenesis[44]. Promoter hypermethylation, and downregulation of TIMP3 expression is observed in various human cancers[43,45-47]. Hypermethylation in PTC occurred in 53% of tumors analyzed and was associated with extrathyroidal invasion (38%), lymph node metastasis (43%), and multifocality (49%).

Death-associated protein kinase (DAPK)

DAPK is a calcium/calmodulin-dependent serine threonine kinase protein with a pro-apoptotic, tumor suppressor function[43,48]. The DAPK gene is silenced by hypermethylated in several human cancers[43,49,50], including thyroid cancer, and its expression has been shown to be a useful marker for cancer prognosis[48]. In addition to aberrant DNA methylation, chromatin immunoprecipitation analysis demonstrated that histone deacetylation of the 5' CpG island is involved in gastrointestinal malignancies[49]. In papillary thyroid cancer, promoter hypermethylation of DAPK was demonstrated in 34% of PTC and was associated with tumor multifocality (51%)[43].

Retinoic acid receptor-β2 (RARβ2)

RARβ2 plays a central role in the regulation of epithelial cells growth and tumorigenesis. Effects of retinoids are mediated by nuclear receptors, RAR-α, RAR-β, and RAR-γ, RXR-α, RXR-β, and RXR-γ which form RXR-RAR heterodimers, which bind to specific DNA sequences, called RAR elements. It is thought that decreased expression of RARs may lead to resistance to retinoid effects[51]. Hypermethylation of RARβ2 was demonstrated in 22% of papillary thyroid cancer, and was not associated with any aggressive clinicopathologic features[43].

Role of the Ras association domain family 1 isoform A (RASSF1A) in tumorigenesis

The Ras association domain family 1 isoform A (RASSF1A) is one of 3 transcripts of the RASSF1 gene on chromosome 3p21.3, derived from alternative splicing[52]. Activation of this tumor suppressor gene is implicated in a number of sporadic cancers. Rassf1A is thought to be an effector of RAS oncoproteins due to its RAS association domain and is involved in regulation of cell cycle progression, apoptosis, and microtubule stability.

Rassf1A functions as a negative regulator of cell proliferation as evidenced by point mutations that inhibit tumor cell growth[53-56]. Rassf1A inhibits G1/S-phase progression; re-expression of RASSF1A in lung and breast tumor-derived epithelial cells results in growth arrest but not apoptosis[55], which correlates with inhibition of cyclin D1 accumulation and subsequent loss of arrest at the RB family cell cycle restriction point, allowing progression to S phase. Regulation of cyclin D1 accumulation by Rassf1A is

independent of the cyclin D1 promoter and occurs through inhibition of mRNA translation[55].

Rassf1A is thought to play a role as a scaffolding protein that assembles effector protein complexes. The death receptor stimulation results in the formation of a complex between Rassf1A and a microtubule-association protein, MAP1. In the absence of Rassf1A, MAP1 exists in an inactive closed conformation in inhibiting its interaction with BAX, a member of the BCL2 family and an important component of apoptotic machinery[57,58]. RassfF1A/MAP1 interaction is enhanced by activate K-Ras and is required for conformational change in BAX, mitochondrial membrane insertion, and maximal apoptosis in response to death receptor stimulation. Rassf1A and MAP1 are also recruited to the TNF-alpha and TRAIL receptor apoptosis complexes in response to their respective cognate ligands [59]. Ectopic expression of Rassf1A enhances apoptosis in breast cancer cells, and knockout by RNA interference in osteosarcoma and breast cancer cells or by gene deletion in Rassf1a-null mouse embryonic fibroblasts specifically impairs death receptor-dependent apoptosis[59-61].

Another scaffolding role for Rassf1A involves stabilization of microtubules, modulated by microtubule-associated proteins (MAPs). MAPs bind directly to tubulin, and have also been shown to bind directly to Rassf1A[60,62]. This modulation of tubulin dynamics may involve Rassf1A in cell motility. Overexpression of Rassf1A inhibits cell migration, and Rassf1A knockout reduces cell-to-cell adhesion [61].

Rassf1A is a mitosis-specific inhibitor of the APC/C (anaphase-promoting complex/cyclosome), a large multisubunit complex that collaborates with ubiquitin-conjugating and ubiquitin-activating enzymes. Ubiquitin-activating enzymes catalyze the

formation of polyubiquitin chains on its protein substrates that targets them for proteasome degradation [60]. The activity of APC/C is thought to be restricted to a specific period in the cell cycle by the spatiotemporal regulation of mitosis by Rassf1A[63]. Rassf1A interacts with Cdc20 and inhibits the APC, preventing degradation of cyclin A and cyclin B until the spindle checkpoint becomes fully operational[63].

The RASSF1A locus is genetically and epigenetically inactivated at high frequency in a variety of solid tumors, conforming to Knudson's two-hit hypothesis[52,64].

Figure 2. Pathways involving RASSF1A[65]. RASSF1A regulates mitosis, the cell cycle and apoptosis in response to mitogenic or apoptotic stimuli. Direct interaction between RASSF1A and microtubule-associated proteins localizes RASSF1A to the microtubules, stabilizing them and, thereby, regulating mitosis. Repression of cyclins A and D1 by RASSF1A results in cell cycle arrest and interactions with CNK1, MST1, Salvador and MOAP1 may allow RASSF1A to modulate apoptosis.

Table 1. Frequency of RASSF1A promoter hypermethylation in various tumor types[65].

Tumor type	**Frequency of hypermethylation**
Lung (SCLC)	88%
Lung (NSCLC)	15 – 39%
Breast	81 – 95 %
Colorectal	20 – 52%
Prostate	99%
Cervical Adenocarcinoma	45%
Esophageal	34%
Gastric	44%
Renal	56 – 915
Hepatocellular	75%
Bladder	30 – 50%
Pancreatic	63%
Ovarian	26 – 30%
Nasopharyngeal	68%
Leukemia	0 – 15%
Neuroblastoma	83%
Thyroid	35 – 71%
Cholangiocarcinoma	67%
Ependymoma	36 – 86%
Glioma	54 – 57%
Hodgkin's lymphoma	65%
Medulloblastoma	79%

Retinoblastoma	59%
Testicular (Seminoma)	40%
Tesicular (Non-seminoma)	83%
Wilm's tumor	54%
Rhabdomyosarcoma	61%
Pheochromocytoma	22%
Head and Neck	15 – 17%
Melanoma	41%

HYPOTHESIS AND SPECIFIC AIMS

We hypothesize that characterization of the PTC "methylome" is likely to provide insights into the molecular pathogenesis of PTC and lay the basis for prevention, improved diagnosis, and classification of thyroid cancer and provide critical insights for individualized medical and surgical treatment of these patients. We aim to

1. Characterize the PTC "methylome" in a discovery cohort of clinically well-characterized patients with both early (Stage I, T1 N0) and late stage PTC (Stage III-IV, T3-4, N1b, M1) via quantitative whole genome DNA methylation analysis in a "discovery set" of human PTC.
2. Analyze hypermethylation, gene silencing and function of selected genes identified during the discovery phase in a larger "verification set" of human PTC.

This approach is likely to identify not only genes showing frequent alterations in methylation patterns in PTC, but also genes and pathways altered in aggressive metastatic PTC. Additional studies will be performed in a larger verification cohort of PTC as well as functional, *in vitro*, studies of genes identified using this novel technology.

MATERIAL AND METHODS

Subjects and Tissues (performed by thesis author)

Papillary thyroid carcinomas (n=50) were acquired from patients diagnosed and surgically treated in the clinical routine at Yale-New Haven Hospital and clinical characteristics are presented in Table 1. All tumors were carefully evaluated and dissected by an experienced endocrine pathologist prior to use in the study and the diagnosis of papillary thyroid carcinoma was unequivocal. Tissues were snap-frozen in OCT using liquid nitrogen and stored in -80ºC. Informed consent and approval by the institutional review board was obtained.

DNA and RNA isolation and Gene sequencing

High molecular weight genomic DNA was isolated from normal and neoplastic thyroid tissue as previously described[66] and gene sequencing was performed by the Yale Keck Facility and analyzed using Genome Studio CodonCode Aligner.

Bisulfite modification of DNA (performed by thesis author)

Genomic DNA (500ng) was bisulphite modified using the EZ DNA Methylation kit (Zymo Research, Orange, CA) according to the instructions from the manufacturer.

Genome-wide DNA methylation profiling (performed by the principal investigator and lab residents)

Bisulfite-modified DNA was analyzed using Infinium HumanMethylation27 BeadChip (Illumina, San Diego, CA). The Infinium HumanMethylation27 BeadChip protocol comprises six steps (whole-genome amplification, fragmentation, hybridization, washing, counterstaining and scanning)[67], which were carried out at the Yale Center for Genome Analysis at Yale University according to the manufacturer's recommendation. Details can be found at http://www.illumina.com/downloads/InfMethylation_AppNote.pdf. The Infinium HumanMethylation27 BeadChip is a novel genome-wide DNA methylation platform that allows interrogation of 27,578 highly informative CpG sites per sample at single-nucleotide resolution[68,69]. The 12-sample Bead Chip features content derived from the NCBI CCDS database (Genome Build 36) and is supplemented with more than 1,000 cancer-related genes[68,69].

Methylation-specific Real-time PCR (MSP) analysis using SYBR Green (performed by thesis author)

To verify the findings from the methylation arrays, selected genes were analyzed using MS-PCR. Methylation-specific primers (table 2) were designed using the Methyl Primer Express software. MethPrimer is based on Primer 3 software and searches an input DNA sequence for potential CpGislands then designs MSP primers around those sites. Semiquantitative PCR was performed on Bio-Rad systems Real-Time PCR systems (Bio Rad Laboratories Inc. Hercules, CA, USA) using SYBR-Green PCR Master Mix

(#4309155) and results were analyzed using StepOne Software v2.1 (Applied Biosystems, Foster City, CA, USA). Commercial methylated, unmethylated, and genomic DNA were used as MSP assay controls and for determining the cut-off for hypermethylation. Percent methylation was calculated using the formula 100/[1+2^(Ct Meth – Ct UnMeth)[1]]

Table 2. Primers used for methylation-specific real-time PCR analysis of gene expression

CpG Island	**Forward primer**	**Reverse primer**
RASSF1A-CpG#1-M	tttcgaagggtgaggtattc	ctcctatctcgaaacgctct
RASSF1A-CpG#1-UM	gggttttgaagggtgaggtattt	atactcctatctcaaaacactct
RASSF1A-CpG#2-M	agaaatacgggtattttcgc	acgaaactaaacgcgctc
RASSF1A-CpG#2-UM	tttagaaatatgggtatttttgt	aaacaaaactaaacacactct
CDKN2B/p15-CpG#1-M	attatagcggatagggggc	cgcctcgctctaacaaaat
CDKN2B/p15-CpG#1-UM	gattatagtggatagggggt	ccacctcactctaacaaaat
CDKN2B/p15-CpG#2-M	gggttttagggtttcgtc	cgcgtaaaatacacacct
CDKN2B/p15-CpG#2-UM	tggggttttagggttttgtt	tacacataaaatacacacct

[1] Ct Meth and Ct UnMeth are the threshold cycles of methylated DNA and unmethylated DNA from the MSP reaction.

Quantitative RT-PCR analysis (performed by thesis author).

Complementary DNA (cDNA) was synthesized using 1 μg of total RNA and iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc. Hercules, CA, USA). Primers (table 3) were designed with Primer3Plus software or obtained from the NCI QPCR primer database. Quantitative real-time PCR was performed on Bio-Rad systems Real-Time PCR systems (Bio Rad Laboratories Inc. Hercules, CA, USA) using assays for CDKN2B, RASSF1A, and GAPDH (All from Applied Biosystems, Foster City, CA). Each cDNA sample was analyzed in triplicate. Standard curves were established for each primer.

Table 3. Primers used for quantitative real-time PCR analysis of gene expression.

Gene	**Forward primer**	**Reverse primer**
RASSF1A	tgggagacacctgacctttc	tgggcaggtaaaaggaagtg
GAPDH	aaggtgaaggtcggagtcaa	aatgaaggggtcattgatgg

Statistical analysis (performed by the principal investigator, lab resident, and thesis author)**.** The BeadChip was scanned on the Illlumina iScan and the resulting files were analyzed with the Beadstudio software (Version 3.2; Illumina). The output of the Beadstudio analysis is a beta-value for each CpG site interrogated. This is a continuous value between 0 and 1 where 0 indicates 0% methylation and 1 indicates 100% methylation at a given CpG site. Therefore, this assay provides quantitative methylation

measurement at the single CpG site level. The calculation of the beta-value is performed as described[67]. Wilcoxon rank test, Student's unpaired t-test, and Fisher exact test were used for statistical evaluation, with $p<0.05$ considered to be significant. All results are expressed as mean ± SEM (standard error of the mean).

RESULTS

Hierarchical clustering of genes with altered DNA methylation profiles in papillary thyroid cancer did not show any relationship to age, gender, tumor size, lymph node status, pathological or clinical TNM stage. However, a distinct DNA methylation profile was noted in BRAF V600E PTC versus BRAF WT PTC, with 24 genes displaying significant hypermethylation in BRAF V600E PTC versus BRAF WT ($p<0.005$) (table 4). These included genes involved in transcriptional regulation and cell cycle control such as CDKN2B/p15 and RASSF1A.

BRAF mutational status was determined by Sanger sequencing of papillary thyroid tumors (figure 3). Twenty-five BRAF V600E PTC and BRAF WT PTC (table 5) were selected for validation of the methylation profile of RASSF1 and analysis of its gene expression. Tumors in the BRAF V600E cohort had the characteristic aggressive phenotypes expected. Extrathyroidal extension, lymphovascular invasion, and lymph node involvement was observed in 34% ($p < 0.005$), 22% ($p < 0.005$), and 48% ($p < 0.0005$) of tumors in the BRAF V600E cohort versus BRAF WT, respectively (table 5). In addition, tumors in the BRAF V600E cohort presented at a higher AJCC stage. 59% of BRAF V600E tumors were AJCC stage III-IV compared to 5% of the BRAF WT tumors ($p < 0.005$). No significant difference in patient age or tumor size was observed.

Hypermethylation observed on the Infinium HumanMethylation27 BeadChip methylation array was validated by methylation-specific quantitative PCR (MS-PCR). One of two CpG islands (CpG#1) in the *Rassf1A* promoter was equally hypermethylated in BRAF V600E PTC and BRAF WT PTC, with an average of 39% and 38% respectively (figure 4). The second CpG island (CpG#2) was differentially methylated

with an average of 73% and 8% ($p<0.0005$) methylation in BRAF V600E PTC and BRAF WT PTC respectively (figure 5). Commercially available fully methylated, fully unmethylated, and genomic DNA used as methylation controls, showed 96%, 1%, and 18% methylation of the *Rassf1A* promoter respectively (figure 6A).Ninety-two % (n=23) of the BRAF V600E tumors were hypermethylated at CpG#2, compared to 12% (n=3) of the BRAF WT tumors (figure 4A).

Two-step quantitative PCR was performed on RNA isolated from all tumors in both cohorts. Gene expression was quantified and Rassf1A expression was normalized to Gapdh. The efficiency of both primers were determined using a standard curve. Normalized *Rassf1A* expression from each BRAF V600E tumor was compared to the average expression in the BRAF WT cohort. Expression of *Rassf1A* was decreased 0.64-fold in BRAF V600E PTC relative to expression in BRAF WT PTC ($p<0.05$).

Promoter hypermethylation of *CDKN2B/p15* was also addressed. Two CpG islands were assessed in the 5'-UTR and intron 1, and neither of these CpG islands were hypermethylated in PTC. 99% and 0% methylation of the *CDKN2B/p15* promoter was seen in the methylated and unmethylated DNA controls, with some variation in the genomic controls. The 5'UTR CpG island showed 8% methylation while the CpG island in intron 1 showed 34% methylation. The average methylation seen in the PTC cohorts at both CpG islands was <2% (data not shown).

Table 4. 24 genes hypermethylated in BRAF V600E PTC versus BRAF WT PTC

Symbol	Name	BRAF V600E PTC beta value[2]	BRAF WT PTC beta value	Delta Beta
FAM113B	Hypothetical protein LOC91523	0.61	0.41	0.20
CD6	CD6 antigen	0.79	0.59	0.20
FLJ45909	Hypothetical protein LOC126432	0.61	0.41	0.20
PTPN7	Protein tyrosine phosphatase; non-receptor type 7 isoform 1	0.70	0.50	0.20
CDKN2B	Cyclin-dependent kinase inhibitor 2B	0.52	0.32	0.20
KLHL6	Kelch-like 6	0.62	0.41	0.20
MS4A1	Membrane-spanning 4-domains; subfamily A; member 1	0.70	0.50	0.20
SEMA3B	Semaphorin 3B isoform 1 precursor	0.72	0.51	0.20
SLAMF8	B lymphocyte activator macrophage expressed	0.56	0.36	0.20
LGP2	Hypothetical protein LOC79132	0.33	0.12	0.20
CD5	CD5 antigen (p56-62)	0.80	0.59	0.21
RASSF1	Ras association domain family 1 isoform A	0.55	0.34	0.21
C16orf54	Chromosome 16 open reading frame 54	0.69	0.49	0.21
CDH5	Cadherin 5; type 2 preproprotein	0.64	0.43	0.21
ACY3	Aspartoacylase (aminocyclase) 3	0.53	0.33	0.21
LAPTM5	Lysosomal associated multispanning membrane	0.55	0.34	0.21

[2] The beta-value or methylation index (MI) for each CpG site corresponds to the ratio of the fluorescence signal from the methylated allele (C) to the sum of the fluorescent signals of the methylated (C) and unmethylated (T) alleles[75]. The methylation status for each detected CpG site ranges between 0.1 (completely unmethylated) to 1 (completely methylated). Genes with a delta beta value ≥ 0.20 (difference between the methylation status in BRAF V600E and BRAF WT) were considered differentially methylated.

	protein 5			
LTA	Lymphotoxin alpha precursor	0.72	0.51	0.21
CREB3L3	cAMP responsive element binding protein 3-like 3	0.39	0.18	0.21
PPP1R16B	Protein phosphatase 1 regulatory inhibitor subunit 16B	0.58	0.37	0.21
LSP1	Lymphocyte-specific protein 1 isoform 1	0.74	0.53	0.21
ZBTB32	Testis zinc finger protein	0.81	0.60	0.21
LAT	Linker for activation of T cells isoform a	0.68	0.47	0.21
LIMD2	Hypothetical protein LOC80774	0.61	0.40	0.22
LY9	Lymphocyte antigen 9 isoform a	0.67	0.45	0.22
RASSF1	Ras association domain family 1 isoform A	0.54	0.32	0.22
VCY	Variable charge; Y-linked	0.73	0.51	0.22
CD79B	CD79B antigen isoform 1 precursor	0.63	0.41	0.22
FLJ33860	Hypothetical protein LOC284756	0.62	0.40	0.22
TMC8	EVIN2	0.76	0.53	0.23
C16orf54	Chromosome 16 open reading frame 54	0.78	0.55	0.23
C17orf62	Chromosome 17 open reading fram 62	0.64	0.40	0.23
RASSF1	Ras association domain family 1 isoform A	0.59	0.36	0.23
GPR114	G-protein coupled receptor 114	0.61	0.37	0.24
CD6	CD6 antigen	0.79	0.55	0.24
RASSF1	Ras association domain family 1 isoform A	0.61	0.37	0.24
C16orf24	Chromosome 16 open reading frame 24	0.56	0.31	0.26

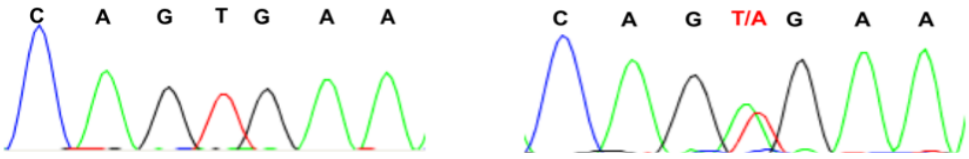

Figure 3. Sanger sequencing of the BRAF T1799A mutation. Left: Homozygous T1799T sequence in BRAF WT PTC. Right: Heterozygous T1799A in BRAF V600E PTC.

Table 5. Twenty-five BRAF V600E PTC and BRAF WT PTC were used for validation of the methylation profile of RASSF1 and analysis of its gene expression. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$

Clinicopathologic characteristics	***BRAF V600E* PTC**	***BRAF WT* PTC**
Age (yrs)	47	50
Tumor size (cm)	1.88	1.24
Classical variant (%)**	91	50
Follicular variant (%)**	9	50
Unifocal (%)	22	20
Multicfocal (%)*	65	40
Extrathyroidal extension (%)**	34	0
Lymphovascular invasion (%)**	22	0
Lymph node metastasis (%)***	48	0
AJCC Stage I – II (%)**	41	95
AJCC Stage III – IV (%)**	59	5

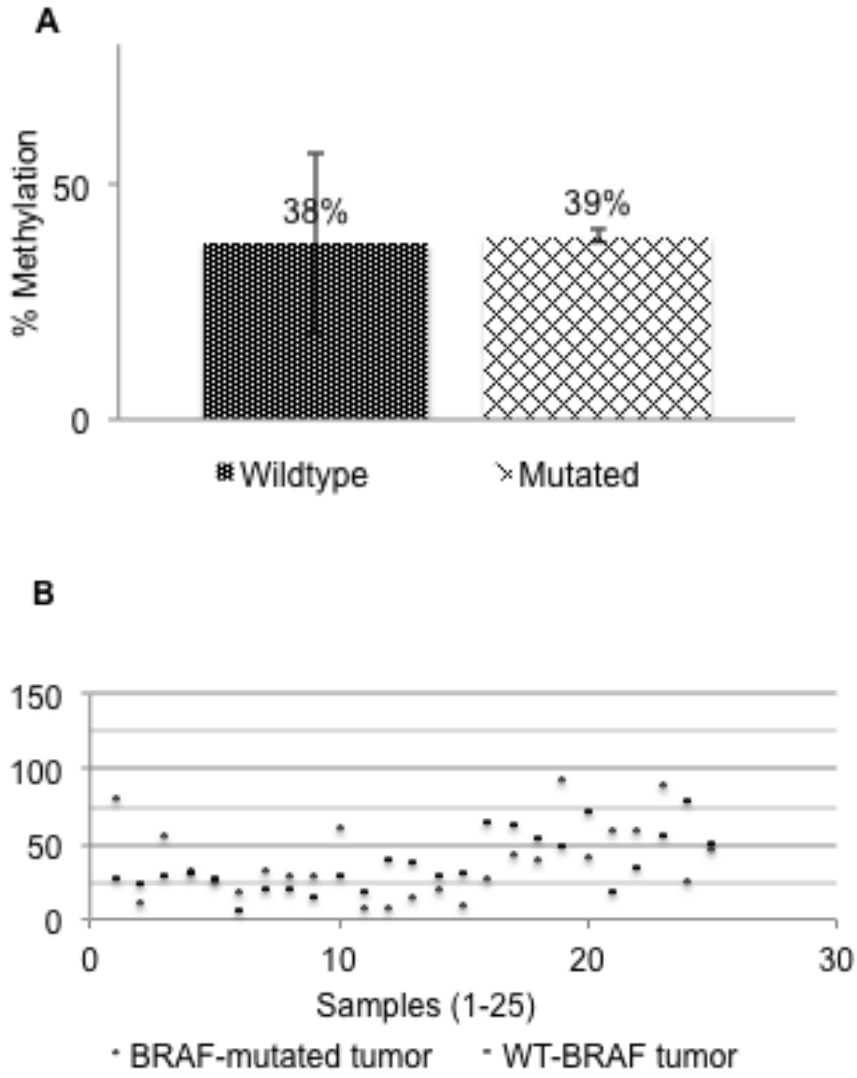

Figure 4. CpG island #1 of the RASSF1A promoter was equally hypermethylated in *BRAF* V600E PTC (mutated) and *BRAF* WT PTC (wildtype). **A.** An average of 38% and 39% methylation was observed in the mutated and wildtype samples respectively. **B.** Methylation percentage of CpG island #1 for each individual tumor in the cohorts. Red dots are *BRAF* V600E (mutated) PTC and blues dots are *BRAF* WT (wildtype) PTC.

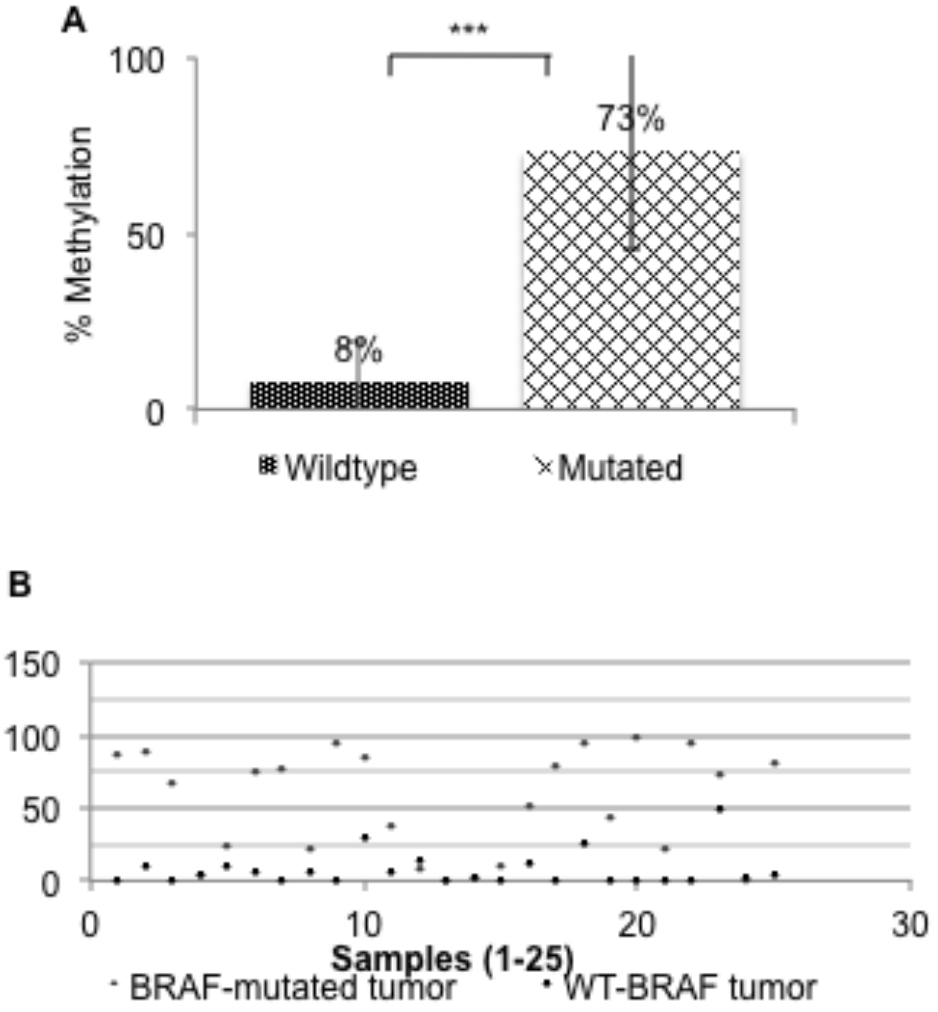

Figure 5. CpG #2 of the RASSF1A promoter was differentially methylated in *BRAF* V600E PTC (mutated) and *BRAF* WT PTC (wildtype). **A.** An average of 73% and 8% methylation was observed in the mutated and wildtype samples respectively. **B.** Shows the methylation percentage of CpG #2 for each individual tumor in the cohorts. Red dots are *BRAF* V600E (mutated) PTC and blues dots are *BRAF* WT (wildtype) PTC. ***$p < 0.0005$

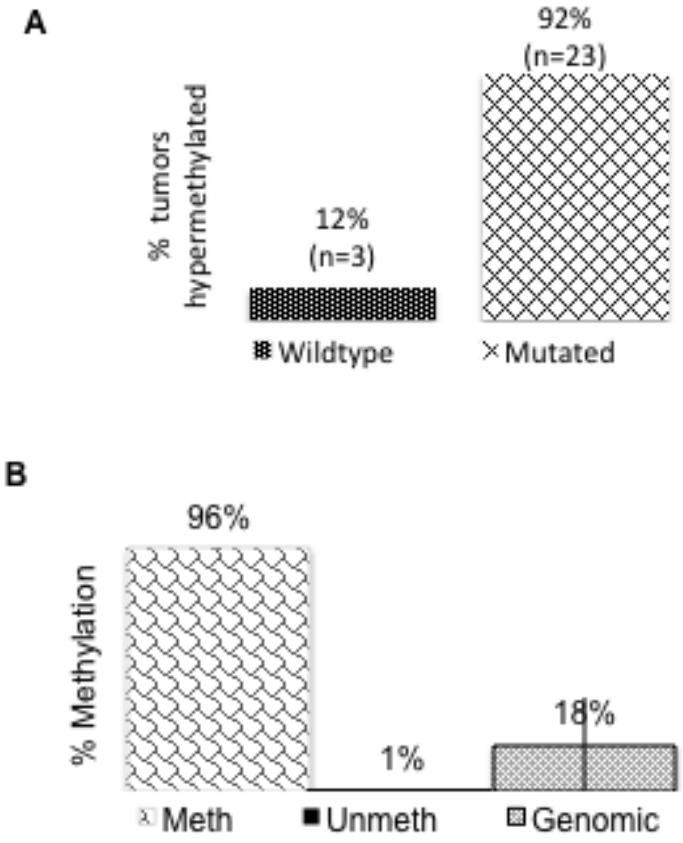

Figure 6. 92% (n=23) of the BRAF V600E tumors were hypermethylated at this CpG island, compared to 12% (n=3) of the BRAF WT tumors. **A.** Percentage of *BRAF* V600E (mutated) PTC and *BRAF* WT (wildtype) PTC hypermethylated. **B.** Shows the methylation percentages of commercially available control DNA. Meth = fully methylated DNA; Unmeth = fully unmethylated DNA; Genomic = genomic DNA. Hypermethylation defined as methylation >18% (observed in genomic DNA).

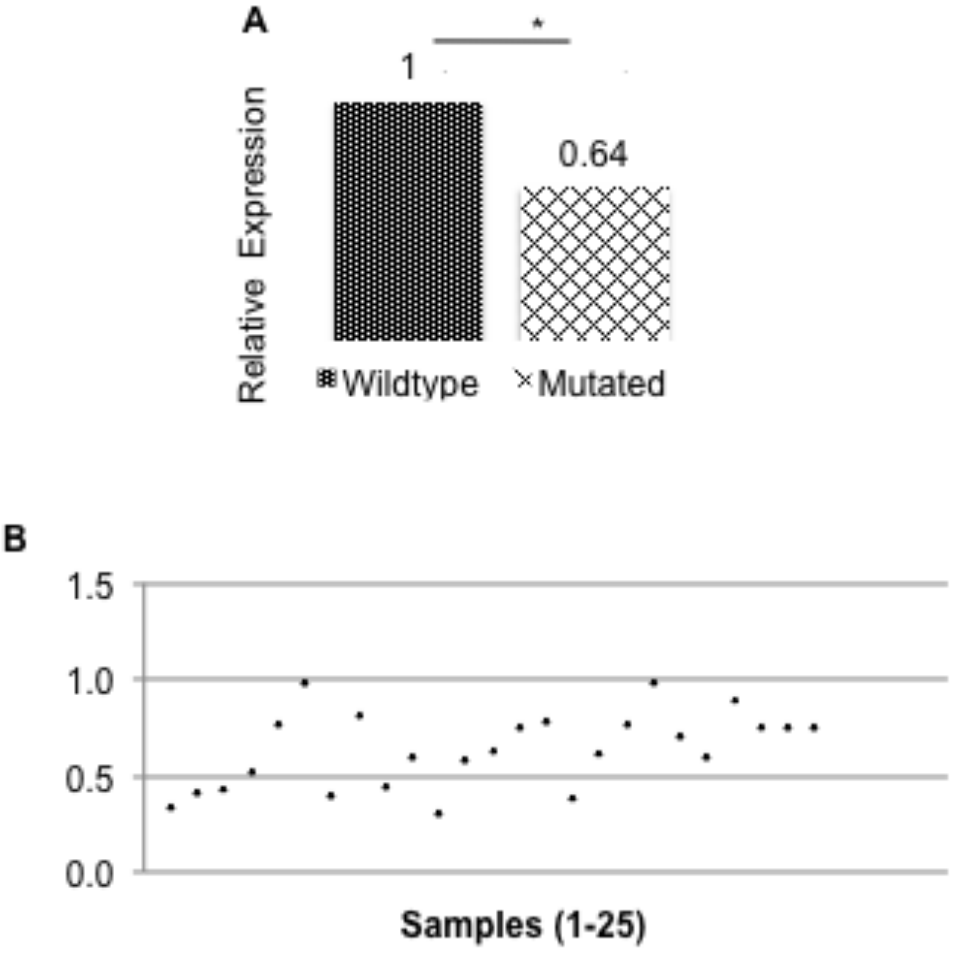

Figure 7. Relative expression of *RASSF1A* was determined by quantitative QPCR analysis, normalized to *GAPDH*. **A.** *RASSF1A* expression was decreased in *BRAF* V600E PTC relative to expression in *BRAF* WT PTC ($p<0.05$). **B.** RASSF1A expression of individual samples in the *BRAF* V600E PTC cohort relative to the mean expression of the *BRAF* WT PTC cohort.

DISCUSSION

The DNA methylation profile of PTC correlates with BRAF mutational status, underscoring its importance in PTC development. Twenty-four genes were significantly hypermethylated in BRAF V600E versus BRAF WT PTC, including genes involved in transcriptional control and cell cycle regulation. One of two CpG islands in the RASSF1A promoter was hypermethylated in 92% of BRAF V600E PTC, with an average of 72% methylation, compared to 12% of BRAF WT PTC (8% average methylation). Hypermethylation of this CpG island appears to correlate with reduced RASSF1A mRNA levels in BRAF V600E PTC. No other clinical or pathological characteristics correlated with the DNA methylation profile of PTC on the genome-wide DNA methylation array performed.

The molecular mechanism of the more aggressive phenotype of BRAF V600E PTC may be related to aberrant DNA methylation of genes involved in transcriptional and cell cycle control. Functional studies assessing the effect of this hypermethylation on RASSF1A gene expression may further determine a cause-effect relationship. In addition to the mRNA analysis performed, protein expression analysis using immunohistochemistry and western blot analysis on PTC tissue would detect any changes in RASSF1A expression in BRAF V600E PTC compared to expression in wildtype tumors. Furthermore, assessing the effect of azanucleoside, a demethylating agent, on RASSF1A gene expression in PTC cell cultures may further determine a cause-effect relation, and would be critical in assessing the possibility of clinically applying a demethylating agent to treatment of aggressive, BRAF V600E PTC.

With improved diagnostic techniques, endocrine tumors are identified much more frequently than in the past. For instance, benign parathyroid tumors occurs in as many as 2.3% of postmenopausal women[70], and primary hyperaldosteronism may be the cause of hypertension in as many as 4.8% of all patients with elevated blood pressure[71]. The molecular genetics of rare inherited endocrine tumor susceptibility syndromes, such as multiple endocrine neoplasia (MEN) type 1 and 2, familial pheochromocytoma syndromes, Carney Complex, and Beckwith-Wiedemann syndrome have all contributed to our understanding of endocrine tumor development[72,73]. Among different tumor types, there exist common pathways that lead to tumorigenesis, such as inactivation of the MEN1 tumor suppressor and activation of the RET proto-oncogene. As in other cancers, it is believed that the vast majority of genetic changes are somatic, i.e. tumor specific mutations acquired during tumor progression[74].

Epigenetic mechanisms, especially aberrant DNA methylation, very likely play an important role in papillary thyroid tumorigenesis. Genome-wide DNA methylation studies in PTC provide a powerful tool to identify disease-causing genes. Additionally, unbiased, systematic analyses of tumor methylomes are likely to identify signaling pathways of importance in cancer development, in general. Analyzing epigenetic alterations in papillary thyroid cancer would help to characterize pathogenesis and may play a critical role in tumor classification and diagnosis. It has recently been shown that there differences in global methylation profiles between prognostic subsets of chronic lymphocytic leukemia (CLL)[75]. The specific silencing of unmethylated tumor suppressor genes was seen in the unmutated IGHV subgroup of CLL, implying a critical role for epigenetic changes during leukemogenesis. Interestingly, patients with immunoglobulin

heavy-chain variable gene (IGHV) unmutated CLL have worse prognoses compared to CLL patients with mutated IGHV genes[75].

These studies may also pave the way for the application of epigenetic therapeutics, by targeted reversal of gene silencing. Azanucleoside drugs are demethylating agents that are currently approved for treatment of myelodyspastic syndrome[19,72]. These function as DNA methyltransferase enzymes, require incorporation into DNA to be effective, and affect the differentiated state. Other nucleoside DNA methylation inhibitors include 5-fluoro-2'-deoxycytidine and zebulamine[76] which are in development. Histone deacetylases[77,78] and histone methyltransferases are another reasonable option for therapeutics. The histone deacetylase SAHA is currently approved by the FDA for treatment of T cell lymphoma[79].

Just as multiple epigenetic events may act in concert to affect a single-cell pathway, it is most likely that epigenetic therapy will involve using multiple drugs that individually affect epigenetic silencing but have synergistic effects. Proposed strategies for the FDA approved epigenetic drugs are as single therapies or in combination as primary or secondary treatment after neo-adjuvant chemotherapy. Lack of specificity may not pose a problem since DNMT inhibitors act only on dividing cells. The drugs preferentially activate genes that have become abnormally silenced in cancer[80]. Moreover, the chromatin structure associated with a pathologically silenced gene may be more susceptible to reactivation than the highly compacted state induced by physiological silencing[19].

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