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Recent Advances in Molecular Biology of Thyroid Cancer and Their Clinical Implications

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Synopsis

Thyroid cancer is the most common endocrine malignancy with a rapid rising incidence in recent years. Novel efficient management strategies are increasingly needed for this cancer. Remarkable advances have occurred in recent years in understanding the molecular biology of thyroid cancer. This is reflected in several major biological areas of thyroid cancer, including the molecular alterations for the loss of radioiodine avidity of thyroid cancer, the pathogenic role of the MAP kinase and PI3K/Akt pathways and their related genetic alterations, and the aberrant methylation of functionally important genes in thyroid tumorigenesis and pathogenesis. These exciting advances in molecular biology of thyroid cancer provide unprecedented opportunities for the development of molecular-based novel diagnostic, prognostic, and therapeutic strategies for this cancer.

Keywords

Thyroid cancer; Iodine; thyroid genes; MAP kinase pathway; PI3K/Akt pathway; mutation; *BRAF* mutation; methylation; molecular marker

Thyroid Cancer and Its Clinical Challenges

Thyroid cancer is the most common endocrine malignancy and its incidence is rapidly rising in recent years in the world (1-3). In the United States, the rise in the incidence of thyroid cancer is the fastest among cancers in many patient populations, particularly women and elder patients of both genders, with an estimated 2008 incidence of 37,340 cases and a prevalence of above 350,000 cases (3,4). The major histological types of follicular cell-derived thyroid cancer are papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC) (1-3,5). Benign thyroid adenoma (BTA) is a common endocrine tumor. Medullary thyroid cancer derived from para-follicular cells is a relatively rare malignancy and will not be discussed here. PTC and FTC are generally differentiated, indolent, and highly curable with current treatments. ATC is an undifferentiated and rapidly lethal thyroid cancer (6,7). In fact, ATC is among the most aggressive and deadly human cancers. There are also poorly differentiated thyroid cancers that have a high incurability and mortality albeit with a better prognosis than ATC. Poorly differentiated thyroid cancer can progress into ATC, and

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both can derive from PTC and FTC or occur *de novo*. Thyroid cancers, particularly poorly differentiated thyroid cancer and ATC, can lose the ability to take up iodide and consequently do not respond to radioiodine treatment (discussed below); they become incurable if they are also surgically inoperable. This is currently a major therapeutic challenge for thyroid cancer and is the main cause of thyroid cancer-related morbidity and mortality.

There are also several diagnostic challenges that are often encountered in the clinical management of thyroid cancer. One is the diagnostic dilemma associated with “indeterminate cytology” on the widely used fine needle aspiration biopsy (FNAB) in the evaluation of thyroid nodules. About 300,000 cases of thyroid nodules, which are mostly BTA, are diagnosed annually in the United States (8). About 20-30% of FNAB cases show “indeterminate” cytological findings, a pattern that has remained essentially unchanged over the last two decades (9,10). These patients currently virtually all pursue thyroid surgery to definitely reveal the nature of the nodules although vast majority of them will surgically prove to have benign nodules. Another diagnostic dilemma in the management of thyroid cancer is the difficulty in monitoring thyroid cancer recurrence using the standard serum thyroglobulin (Tg) testing when Tg antibodies are positive, which occurs in about 20-30% of cases, rendering the testing results unreliable in these patients (11). Careful risk stratification is a key step in the decision making for appropriate surgical and medical managements of patients with thyroid cancer. This risk evaluation is conventionally based on clinicopathological factors, which are often unreliable and are mostly unavailable prior to thyroid surgery. The recent advances in understanding the molecular biology of thyroid cancer, particularly in the several areas discussed below, shine great promises on the development of novel molecular-based strategies to effectively tackle these diagnostic, prognostic, and therapeutic obstacles of thyroid cancer.

Impaired Iodide Metabolism in Thyroid Cancer

The unique and fundamental function of the follicular epithelial thyroid cells is to utilize iodide as a substrate to synthesize thyroid hormones to meet the normal metabolism of the body, a process that involves several thyroid-specific iodide-handling protein molecules (12). In this process, iodide is transported from the blood stream into the thyroid cell through the sodium/iodide symporter (NIS) in the basal membrane, followed by its transportation into the follicular lumen through such transporters as pendrin (also called SLC26A4) in the apical membrane of the cell. Through thyroid peroxidase (TPO), iodide is oxidized and organified into thyroglobulin (Tg) through iodination of tyrosine residues in Tg for the formation of thyroid hormones. The thyroid-specific transcription factors TTF-1, TTF-2, and Pax-8 play an important role in the regulation of these thyroid genes. The entire process is up-regulated by the thyroid-stimulating hormone (TSH) that acts by binding to the TSH receptor (TSHR) on the cell membrane.

Expression of thyroid molecules, such as TSHR, NIS, TPO, Tg, and pendrin is often lost in thyroid cancers (13-18), resulting in impaired or loss of the ability of thyroid cancer cells to concentrate radioiodine. Consequently, such cases of thyroid cancer will fail radioiodine ablation therapy, the mainstay of medical treatment for this cancer. As stated above, this represents a major challenge in the current treatment of thyroid cancer, particularly in poorly differentiated thyroid cancer and ATC (6,7). The specific molecular mechanism underlying the silencing of thyroid-specific genes in thyroid cancer is largely unclear. Our group demonstrated that *BRAF* mutation was closely associated with loss of radioiodine avidity in PTC (further discussed below) (19). Recent studies from our and other groups demonstrated that thyroid-specific genes could be silenced by induced expression of *BRAF* mutant and the consequent over-activation of the Ras→Raf→MEK→MAP kinase/ERK pathway (MAP kinase pathway) in thyroid cell lines (20,21) and the genes could be re-expressed by removing the activated MAP kinase pathway signaling (21). We also showed that methylation of the

TSHR gene was a mechanism in its silencing promoted by *BRAF* mutant (21). Very recently, we showed that the PI3K/Akt pathway may also play an important role in the regulation of thyroid genes (Hou et al, unpublished data). With this recent knowledge on the molecular events associated with thyroid-specific genes in thyroid cancer, it can now be expected that unique treatment strategies targeting at these molecular abnormalities may be developed to restore the radioiodine avidity of thyroid cancer.

MAP Kinase Signaling Pathway and *BRAF* Mutation in Thyroid Cancer

MAP kinase pathway is a classical conserved intracellular signaling pathway that plays a fundamental role in cell proliferation, differentiation, apoptosis, and survival and, when aberrantly activated, tumorigenesis (22,23). In thyroid cancer, *RET/PTC* rearrangement is a common activator of the MAP kinase pathway (24,25). Activating *Ras* mutations, which can activate the MAP kinase pathway, are also common in thyroid cancer (26). *BRAF* mutation is a major cause of aberrant activation of the MAP kinase pathway in human cancers (27). Among the three known Raf kinases, A-Raf, B-Raf (*BRAF*), and C-Raf, *BRAF* is the most potent activator of the MAP kinase pathway (22). The T1799A point *BRAF* mutation accounts for more than 90% of the more than 40 mutations identified in the *BRAF* gene (28). This mutation causes a V600E amino acid change in the *BRAF* protein, resulting in constitutive and oncogenic activation of the *BRAF* kinase (27,28).

Discovery and characterization of the T1799A *BRAF* mutation in thyroid cancer represent one of the most exciting advances in the molecular biology of thyroid cancer in recent years (29, 30). In fact, this mutation is the most common known genetic alteration in thyroid cancer. A few other activated *BRAF* mutants are only rarely found in thyroid cancer. These include the *BRAF* K601E (31), AKAP9-*BRAF* (32), *BRAF* V600E+K601del (33,34), *BRAF* V599ins (35), and V600D+FGLAT601-605ins, which results from an insertion of 18 nucleotides at nucleotide T1799 (34). Thus, the T1799A mutation is the most common and virtually the only *BRAF* mutation identified in thyroid cancer (hereafter referred to as “*BRAF* mutation”). Our previous studies showed that *BRAF* mutation was not a germline mutation in familial non-medullary thyroid cancers (36,37), and, as a somatic genetic alteration, occurs exclusively in PTC and PTC-derived ATC, with an average prevalence of about 45% in the former and 25% in the latter; it does not occur in FTC or other types of thyroid tumors (29,30). Transgenic mouse model (38) and our recent cell line and xenograft tumor studies (39,40) demonstrated the tumorigenic ability of the *BRAF* mutation and its requirement to maintain cancer cell growth and proliferation. Numerous studies demonstrated an association of *BRAF* mutation with aggressive clinicopathological outcomes, including tumor invasion, metastasis, and recurrence of PTC (29,30). We also demonstrated an interesting association of *BRAF* mutation with loss of radioiodine avidity in recurrent PTC and its failure to be cured (19). This is consistent with our finding of *BRAF* mutant-promoted silencing of thyroid iodide-handling genes and the reversal of this process by silencing the expression of *BRAF* mutant in thyroid cells as discussed above (21). Numerous studies demonstrated a close association of *BRAF* mutation with de-differentiation of PTC as reflected by decreased expression of thyroid-specific genes in PTC, including *NIS* (20,41,42), *TPO* (41-45), *pendrin* (45), and *Tg* (41). Therefore, *BRAF* mutation is a novel powerful molecular prognostic marker for poorer prognosis of thyroid cancer (30). We recently demonstrated that testing of this marker on preoperative FNAB specimens can preoperatively predict lymph node metastasis, extrathyroidal invasion, and tumor recurrence of PTC and is therefore uniquely helpful in guiding the initial surgical and medical treatments (Xing et al, 2008, unpublished data).

PI3K/Akt Signaling Pathway and Genetic Alterations in Thyroid Cancer

The PI3K/Akt pathway in human cancers

Like the MAP kinase pathway, the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway (PI3K pathway) plays a fundamental role in the regulation of cell growth, proliferation and survival and in human tumorigenesis (46,47). Among the several classes of PI3Ks, class I is the best characterized and is composed of heterodimers of a regulatory subunit, particularly p85, and one of the several p110 catalytic subunits. The α -type (PIK3CA) and β -type (PIK3CB) p110 subunits are widely expressed in different tissues whereas other types of p110 subunits are only expressed in limited tissues. PIK3CA and PIK3CB belong to class IA that is activated by receptor tyrosine kinases. The p110 subunits contain a binding site for the regulatory subunit, through which various signals can be integrated from membrane receptors and activate the catalytic subunits of PI3K. The p110 catalytic subunits also contain a Ras-binding site through which Ras is involved in the PI3K/Akt signaling. Upon activation by signaling from various membrane growth factor receptors, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular epithelial growth factor (VEGF) receptor, c-MET, and c-KIT, the p110 catalytic subunits (PIK3CA and PIK3CB) phosphorylate phosphatidylinositol-4,5-bisphosphate to produce phosphatidylinositol-3,4,5-trisphosphate (PIP₃), which localizes the Ser/Thr kinase Akt to cell membrane where it becomes phosphorylated and activated by the phosphoinositide-dependent kinases (PDK), particularly PDK-1. There are three types of Akts: Akt-1, Akt-2 and Akt-3. Activated Akt phosphorylates down-stream protein effectors and amplifies the signaling cascade, promoting cell proliferation and inhibiting apoptosis. Signaling of the PI3K/Akt pathway is antagonized by the tumor suppressor gene *PTEN* product, PTEN, which is a phosphatase that dephosphorylates PIP₃, hence terminating the signaling of the PI3K/Akt pathway (48).

The *PIK3CA* gene frequently harbors mutations and amplifications in human cancers (49,50). Genetic alterations, including mutation, deletion and aberrant methylation are common mechanisms in the inactivation of the *PTEN* gene in human cancers. Mutations were also found in the *EGFR*, *PDGFR*, *c-MET*, *KIT*, *PDK-1*, and *Akt-2* genes in the PI3K/Akt pathway in certain human cancers, usually in the kinase domains of these proteins (51,52). In addition to the *PIK3CA* amplification, genomic amplifications were found in the *Akt-2* (53), *EGFR* (54), and *PDGFR α* genes (54) in human cancers. Thus, genetic alterations are important driving forces for the aberrant signaling of the PI3K/Akt pathway.

The PI3K/Akt pathway in thyroid cancer

Previous studies showed common activation of the PI3K signaling in thyroid cancers (16,55). Among the three isoforms of Akt, Akt-1 and Akt-2 were the most abundant and important in thyroid cancer (16). We first reported genomic copy gain and amplification of the *PIK3CA* in thyroid tumors, particularly FTC and ATC (56-58). We and others have also shown that *PIK3CA* mutation is particularly common in ATC and is relatively uncommon but can occur in differentiated thyroid cancer (55-58). *Ras* mutation is commonly found in thyroid tumors, particularly FTC and BTA (26). We have previously analyzed a number of genetic alterations in the PI3K pathway, including *PIK3CA* mutation and amplification, *Ras* mutation, and *PTEN* mutation in various thyroid tumors, and found a relatively high prevalence of them, particularly in FTC and ATC (57,58). Coexistence of some of these genetic alterations and their coexistence with *BRAF* mutation were more frequently seen in aggressive thyroid cancers, particularly ATC (57). We have recently expanded these studies to a large panel of genetic alterations, including mutations and genetic amplifications, in about 20 genes in this pathway and found at least one genetic alteration in 46/48 (96%) ATC and co-existence of two or more genetic alterations in 37/48 (77%) ATC (59). Interestingly, genetic alterations that could activate both the MAP kinase and PI3K pathways were found in most (81%) ATC, which was

in good correlation with elevated phosphorylation of ERK and Akt. These data provide the strongest genetic evidence for an extensive role of dual involvement of the MAP kinase and PI3K pathways in the pathogenesis of ATC, supporting a recent hypothesis that targeting multiple signaling pathways, particularly the MAP kinase and PI3K/Akt pathways, may be an effective and necessary therapeutic strategy for thyroid cancer (30).

Aberrant Gene Methylation in Thyroid Cancer

Gene methylation

Gene methylation represents an epigenetic modification of DNA, involving the addition of a methyl group to the fifth carbon of the cytosine residue in a CpG dinucleotide. The regions rich in CpG dinucleotides, termed “CpG islands”, are usually located in the 5'-flanking region of a gene, including the promoter and the first exon areas. Gene methylation, particularly that near the transcription start site, is closely associated with chromatin remodeling and usually silences the gene (60,61). One mechanism in gene silencing associated with methylation is the recruitment of DNA methyl-binding transcription repressors that block the binding of the gene with its transcriptional machinery. DNA methylation may also directly affect the binding of the gene with transcription factors. This is well exemplified by the case of the *TSHR* gene in a rat thyroid cell line in which the transcription factor GA was able to normally bind the non-methylated promoter but could not bind the methylated promoter of the gene (62). DNA methylation is an important physiological mechanism in the regulation of gene expression, particularly during embryogenesis, and is normally present in genes on the inactive X chromosome and imprinted genes. Normally expressed genes, such as the “housekeeping” genes, are protected from methylation, which is critical to life. Aberrant gene methylation occurs frequently in human cancers, including thyroid cancer, leading to inappropriate silencing of genes, particularly tumor suppressor genes (60,61,63).

Aberrant gene methylation in thyroid cancer

Our and other groups have documented aberrant methylation of a large number of tumor suppressor and thyroid-specific genes in thyroid cancers (63). Of particular interest is the several classical tumor suppressor genes, including those for tissue inhibitor of metalloproteinase-3 (TIMP3), death-associated protein kinase (DAPK), SLC5A8, and retinoid acid receptor-beta-2 (RAR β 2), whose methylation was associated with tumor aggressiveness and *BRAF* mutation in PTC in our studies (64). Therefore, as in other human cancers, methylation-mediated gene silencing is an important mechanism in thyroid tumorigenesis (63). In contrast, our previous study demonstrated that the *RASSR1A* methylation was inversely associated with *BRAF* mutation in PTC (65), suggesting that epigenetic disruption of this tumor suppressor gene may play a role in thyroid tumorigenesis through signaling pathways other than the MAP kinase pathway. We recently similarly demonstrated an association of aberrant methylation of DNA repair genes, particularly the *hMLH1* gene, with *BRAF* mutation and aggressiveness of PTC (66). Very recently, we also demonstrated that aberrant methylation of the *PTEN* gene was associated with activating genetic alterations in the PI3K/Akt pathway in thyroid tumors, suggesting a self-enhancing mechanism for the PI3K/Akt signaling through the epigenetic silencing of *PTEN* gene as a consequence of activation of this pathway by genetic alterations (67). Aberrant methylation of thyroid iodide-handling genes, including *NIS* (14), *TSHR* (68-70), *SLC26A4* (71), and *Tg* (72) provides an explanation for the commonly seen silencing of these genes in thyroid cancer. Using a variety of molecular biological approaches, including siRNA and Lucifer-reporting system, we demonstrated that the *TSHR* gene was silenced partially through the *BRAF* mutation-promoted MAP kinase pathway signaling involving methylation of the *TSHR* promoter (21). We also demonstrated that methylation and silencing of the *TSHR* gene were partially reversible by suppressing the MAP kinase pathway either using MEK inhibitor or *BRAF* siRNA. Therefore, functional disruption of an important

gene through epigenetic methylation can be directly linked to the aberrant activation of a major signaling pathway in thyroid cancer. This is consistent with a previous hypothesis that “cross talk” between genetic and epigenetic alterations may occur in thyroid cancer through aberrant signaling of major molecular pathways (29). These results should provide a molecular basis for therapeutic strategies using demethylating agents for thyroid cancer. The DNA methylation markers, which can be easily detected in serum and thyroid FNAB specimens, hold great potential as novel diagnostic and prognostic molecular markers for thyroid cancer as we demonstrated recently (64,73 and Xing et al, data not shown).

Summary

Thyroid cancer is the most common endocrine malignancy associated with several major diagnostic and therapeutic challenges. The recent advance in understanding the molecular biology of this cancer provides opportunities for the development of molecular-based strategies to tackle these challenges. This is particularly reflected in the following several areas: 1) Discovery of the aberrant silencing of thyroid iodide-handling genes through aberrant gene methylation coupled to major signaling pathways, such as the MAP kinase pathway, represents a major step in understanding the molecular defects associated with the loss of radioiodine avidity of thyroid cancer. It may now be possible to develop therapeutic strategies targeting at these molecular abnormalities to restore the radioiodine avidity of thyroid cancer. 2) Discovery of novel genetic alterations in the MAP kinase pathway, particularly the *BRAF* mutation, represents a major breakthrough in understanding the molecular mechanism of PTC pathogenesis. It represents not only a novel effective therapeutic target but also a valuable molecular marker that can even be examined preoperatively on FNAB specimens for risk estimation for thyroid cancer. This is expected to have a major impact on surgical and medical managements of thyroid cancer through achieving better risk stratification. 3) Discovery of the genetic alterations in the PI3K/Akt pathway, particularly their high prevalence and co-existence with genetic alterations in the MAP kinase pathway, represents another major advance in understanding the molecular mechanism of thyroid cancer. This provides a strong genetic basis for an extensive role of the PI3K/Akt pathway and the role of the dual involvement of this and the MAP kinase pathways in thyroid tumorigenesis. Consequently, therapeutic strategies dually targeting the two pathways can be tested for the effective treatment of thyroid cancer. 4) Epigenetic gene alterations, particularly aberrant gene methylation, is another fundamental molecular mechanism in thyroid cancer pathogenesis. Aberrant gene methylation-related molecular abnormalities may not only represent potential therapeutic targets but also represent promising novel diagnostic and prognostic molecular markers for thyroid cancer. Given these exciting advances in understanding the molecular biology of thyroid cancer in recent years, we are now at the verge of the development of novel diagnostic, prognostic and therapeutic strategies for this common endocrine malignancy.

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