Molecular genetics and diagnosis of thyroid cancer

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Abstract | Thyroid cancer is a common type of endocrine malignancy, and its incidence has been steadily increasing in many regions of the world. Initiation and progression of thyroid cancer involves multiple genetic and epigenetic alterations, of which mutations leading to the activation of the MAPK and PI3K–AKT signaling pathways are crucial. Common mutations found in thyroid cancer are point mutation of the *BRAF* and *RAS* genes as well as *RET/PTC* and *PAX8/PPARγ* chromosomal rearrangements. The mutational mechanisms seem to be linked to specific etiologic factors. Chromosomal rearrangements have a strong association with exposure to ionizing radiation and possibly with DNA fragility, whereas point mutations probably arise as a result of chemical mutagenesis. A potential role of dietary iodine excess in the generation of *BRAF* point mutations has also been proposed. Somatic mutations and other molecular alterations have been recognized as helpful diagnostic and prognostic markers for thyroid cancer and are beginning to be introduced into clinical practice, to offer a valuable tool for the management of patients with thyroid nodules.

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Introduction

Thyroid cancer is the most common malignancy of endocrine organs. The vast majority of thyroid tumors arise from thyroid follicular epithelial cells, whereas 3–5% of cancers originate from parafollicular or C cells (Table 1). The follicular cell-derived cancers are further subdivided into well-differentiated papillary carcinoma and follicular carcinoma, poorly differentiated carcinoma (also known as insular carcinoma) and anaplastic (undifferentiated) carcinoma.[1,2] Follicular adenoma is a benign tumor that may serve as a precursor for some follicular carcinomas. Less-differentiated thyroid cancers, namely poorly differentiated carcinoma and anaplastic carcinoma, can develop *de novo*, although many of them arise through the process of stepwise dedifferentiation of papillary and follicular carcinomas (Figure 1).

The worldwide incidence of thyroid cancer has been steadily increasing and has almost tripled over the past 30 years in the US and in other industrialized countries.[3–6] The increased incidence is limited to the papillary type of thyroid cancer and is mostly due to tumors of small size (<1 cm), although large papillary cancers also contribute. The increase in thyroid cancer incidence is generally believed to result, to a considerable extent, from increased access to high-resolution imaging (particularly ultrasonography) and increased use of fine-needle aspiration (FNA) biopsy of small nodules, as well as progressively decreasing stringency of histopathologic criteria applied to the diagnosis of papillary cancer during the past 10–15 years. However, whether these factors can account entirely for this continuous trend, or whether other factors also contribute, remains unknown. Ionizing radiation is a well-known risk factor for thyroid cancer; therefore, concerns remain that the rising incidence might, in part, be due to the wider use of medical radiation and increasing exposure to radiation as a result of nuclear power accidents such as that in Chernobyl.

Thyroid cancer typically occurs in thyroid nodules, which are common and can be detected by palpation and imaging in a large proportion of adults, particularly those of increased age.[7–10] Most thyroid nodules are benign, and the clinical challenge is to accurately and rapidly identify those nodules that harbor cancer. Sampling of thyroid nodules using FNA biopsy with subsequent cytologic examination of collected cells is the most accurate and widely used diagnostic tool at this time. It provides a definitive diagnosis of a malignant or benign nodule in most cases. However, a conclusive diagnosis can not be obtained by use of FNA cytology for about 25% of all nodules,[8,11–14] which hampers the clinical management of patients with these nodules. New diagnostic approaches for such nodules are needed.

Knowledge of genetic alterations occurring in thyroid cancer has rapidly expanded in the past decade. This improved knowledge has provided new insights into thyroid cancer etiology and has offered novel diagnostic tools and prognostic markers that enable improved and personalized management of patients with thyroid nodules. This Review focuses on genetic alterations in follicular cell-derived thyroid cancers.

Genetic alterations in thyroid cancer

Similar to other cancer types, thyroid cancer initiation and progression occurs through gradual accumulation

Competing interests
The authors declare no competing interests.

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Key points

- Activation of MAPK and PI3K–AKT signaling pathways is important for thyroid cancer initiation and progression
- Common mutational mechanisms in thyroid cancer are point mutations, such as those in the *RAS* and *BRAF* genes, and chromosomal rearrangements, such as *RET/PTC* and *PAX8/PPARγ*
- *RET/PTC* and *BRAF/AKAP9* chromosomal rearrangements have strong correlation with radiation exposure; *RET/PTC* can also develop via induction of chromosome fragility
- Association between *BRAF* point mutations and high iodine intake or exposure to chemical elements present at high levels in volcanic areas has been proposed
- Mutational markers can be used to improve cancer diagnosis in fine-needle aspiration samples from thyroid nodules and to aid tumor prognostication

of various genetic and epigenetic alterations, including activating and inactivating somatic mutations, alteration in gene expression patterns, microRNA (miRNA) dysregulation and aberrant gene methylation. Among these alterations, most of the data that have accumulated relate to somatic mutations, many of which occur early in the transformation process and are essential for cancer development. Thyroid cancer represents a type of neoplasia in which critical genes are frequently mutated via two distinct molecular mechanisms: point mutation or chromosomal rearrangement. The former is a result of single nucleotide change within the DNA chain, whereas the latter represents a large-scale genetic abnormality with breakage and fusion of parts of the same or different chromosomes. Importantly, a growing body of evidence suggests that these two distinct mutational mechanisms are associated with specific etiologic factors involved in thyroid carcinogenesis.

Somatic mutations

Most mutations in thyroid cancer involve the effectors of the MAPK pathway and the PI3K–AKT pathway (Figure 2). MAPK activation is crucial for tumor initiation. The mutated genes that affect these pathways encode cell-membrane receptor tyrosine kinases RET and NTRK1 and intracellular signal transducers BRAF and RAS. These typically mutually exclusive mutations occur in approximately 70% of patients with papillary thyroid carcinomas and are associated with particular clinical, histopathological and biological tumor characteristics (Figure 3).[15–18] In follicular thyroid cancer, in addition to mutation of *RAS*, another common event is *PAX8/PPARγ* rearrangement. Thyroid cancer progression and dedifferentiation involves a number of additional mutations that affect the PI3K–AKT pathway and other cell signaling pathways.

RET/PTC and TRK rearrangements

RET/PTC is a chromosomal rearrangement found in papillary thyroid cancer.[19] As a result of the rearrangement, a portion of the *RET* gene is fused to one of several possible partner genes. All chimeric genes contain the portion of *RET* that encodes the intact tyrosine kinase domain of the RET protein fused to an active promoter of another gene that drives the expression and ligand-independent dimerization of the RET/PTC protein, which leads to chronic stimulation of MAPK signaling and tumorigenesis in thyroid cells.[20–22] Two of the most common rearrangement types are *RET/PTC1* and *RET/PTC3*, in which *RET* is fused to either *CCDC6* (also known as *H4*) or *NCOA4* (also known as *ELE1* or *RFG*), respectively.[23,24] Both of these rearrangement types are paracentric, intrachromosomal inversions, as all fusion partners reside on the long arm of chromosome 10.[25,26] By contrast, *RET/PTC2* and nine more recently discovered types of *RET/PTC* rearrangements are all interchromosomal rearrangements formed by *RET* fusion to genes located on different chromosomes.[27–35]

The prevalence and specificity of *RET/PTC* rearrangements for papillary thyroid cancer varies dramatically in

Table 1 | Types of thyroid cancer and their mutational profiles

Characteristics	Papillary carcinoma	Follicular carcinoma	Poorly differentiated carcinoma	Anaplastic (undifferentiated) carcinoma	Medullary carcinoma
Cell type	Follicular	Follicular	Follicular	Follicular	C cell
Main histopathologic variants	Classic papillary type, microcarcinoma, follicular variant, tall-cell variant	Conventional type, oncocytic (Hurthle cell) type	–	–	–
Prevalence (%)	80–85	10–15	<2	1–2	3–5
Frequency of familial forms (%)	5	5	0	0	15–30
Typical route of spread	Local lymph-node metastasis	Hematogenous metastasis, typically to bones and lungs	Invasive local growth, lymph-node and hematogeneous metastases	Invasive local growth, lymph-node and hematogeneous metastases	Lymph-node and hematogeneous metastases
10-year survival (%)	95–98	90–95	~50	<10	60–80
Common mutations and their prevalence (%)	*BRAF* 40–45 *RAS* 10–20 *RET/PTC* 10–20 *TRK* <5	*RAS* 40–50 *PAX8/PPARγ* 30–35 *PIK3CA* <10 *PTEN* <10	*RAS* 20–40 *TP53* 20–30 *BRAF* 10–20 *CTNNB1** 10–20 *PIK3CA* 5–10 *AKT1* 5–10	*TP53* 50–80 *CTNNB1** 5–60 *RAS* 20–40 *BRAF* 20–40 *PIK3CA* 10–20 *PTEN* 5–15 *AKT1* 5–10	Familial forms: *RET* >95 Sporadic: *RET* 40–50 *RAS* 25

*The gene that encodes β-catenin.

reported series of patients.[36,37] In part, this variation is due to true differences in the prevalence of this alteration in papillary thyroid cancer in specific age groups and in individuals exposed to ionizing radiation. Mostly, however, the variation exists because of the heterogeneous distribution of this rearrangement within the tumor and the various sensitivities of the detection methods used. *RET/PTC* rearrangement can be present in a large proportion of tumor cells and detected by multiple methods (clonal *RET/PTC*) or occur in a small fraction or single cells within the lesion and be detectable only by ultrasensitive detection techniques (non-clonal *RET/PTC*).[38,39]

Clonal *RET/PTC* rearrangement occurs in 10–20% of papillary thyroid carcinomas and is specific for this tumor type,[19,39] whereas non-clonal *RET/PTC* have been reported with a significantly higher prevalence in papillary carcinomas and also in a variety of other thyroid tumors and benign lesions.[36] For example, whereas studies that have used Southern blot analysis and regular sensitivity reverse transcriptase PCR found no *RET/PTC* rearrangements in benign thyroid tumors,[19,40–42] other analyses based on highly sensitive detection methods have identified the presence of *RET/PTC* in 10–45% of thyroid adenomas and in other benign nodules and non-neoplastic thyroid lesions.[43–49] These *RET/PTC* rearrangements are non-clonal, as they occur in a small proportion of cells within the tumor nodule or even in single non-neoplastic thyroid cells.

Chromosomal rearrangements involving another receptor tyrosine kinase gene, *NTRK1,* also occur in papillary thyroid carcinomas, although with a significantly lower prevalence than *RET/PTC* rearrangements. The *NTRK1* gene resides on chromosome 1q22 and can be fused to at least three different partner genes located on the same or different chromosomes.[50–52] Rearrangements of the *NTRK1* gene, known as *TRK* rearrangements, have been reported to occur in up to 10–15% of papillary thyroid carcinomas in some series of patients,[53–55] although the prevalence of this rearrangement in papillary carcinomas from many geographical areas is probably <2–5%.

RAS mutations

Human *HRAS*, *KRAS* and *NRAS* genes encode highly related G-proteins that reside at the inner surface of the cell membrane and transmit signals arising from cell-membrane receptor tyrosine kinases and G-protein-coupled receptors along the MAPK, PI3K–AKT and other signaling pathways. Activating point mutations typically affect codons 12, 13 and 61 of the *RAS* genes. In thyroid cancer, *NRAS* codon 61 and *HRAS* codon 61 mutations are most common. *RAS* mutations are found in a variety of thyroid tumors, including 10–20% of papillary carcinomas, 40–50% of follicular carcinomas and 20–40% of poorly differentiated and anaplastic carcinomas.[56–62]

Among papillary carcinomas, virtually all tumors that harbor a *RAS* mutation grow forming neoplastic follicles and no papillary structures and are, therefore, diagnosed as the follicular variant of papillary carcinoma.[15,63] *RAS* mutations are also found in 20–40% of benign follicular adenomas.[57,58,60] The finding of this mutation in benign adenomas as well as in follicular-patterned carcinomas suggest that *RAS*-positive follicular adenomas may serve as a precursor for *RAS*-positive follicular carcinomas and the follicular variant of papillary carcinomas. Furthermore, *RAS* mutations may predispose well-differentiated cancers to dedifferentiation and anaplastic transformation.[64–67]

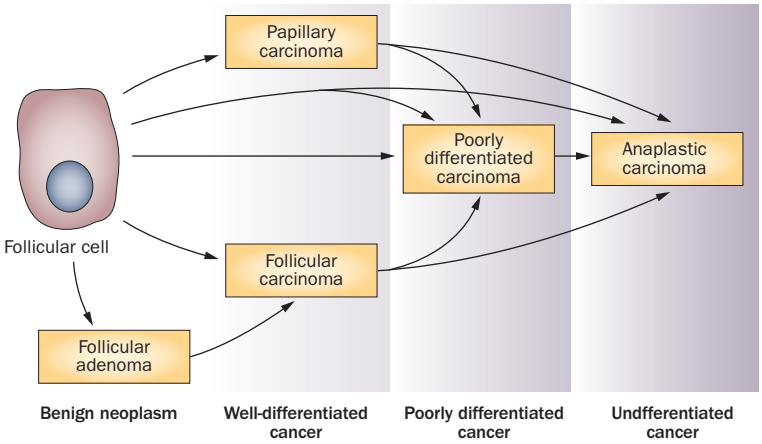

Figure 1 | Scheme of step-wise dedifferentiation of follicular cell-derived thyroid cancer. Adapted from *Expert Reviews of Molecular Diagnostics* **8** (1), 83–89 (2008) with permission from Expert Reviews Ltd.

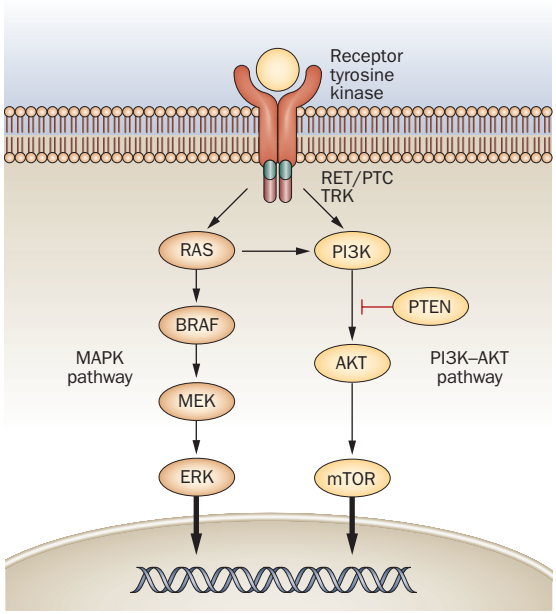

Figure 2 | The main signaling pathways involved in thyroid carcinogenesis are the MAPK and PI3K–AKT pathways. These pathways are involved in propagation of signals from various cell membrane receptor tyrosine kinases into the nucleus, and they regulate multiple cell processes including proliferation, differentiation and survival. Activation of the MAPK pathway by oncogenic stimuli such as mutated BRAF, RAS or the chimeric fusion proteins RET/PTC and TRK is a common tumor initiating event in well differentiated papillary carcinoma and in some follicular carcinomas. Mutations involving the effectors of the PI3K–AKT pathway such as the PI3K subunit PIK3CA, AKT1 and PTEN are found more frequently in follicular carcinomas and in less differentiated types of thyroid cancer.

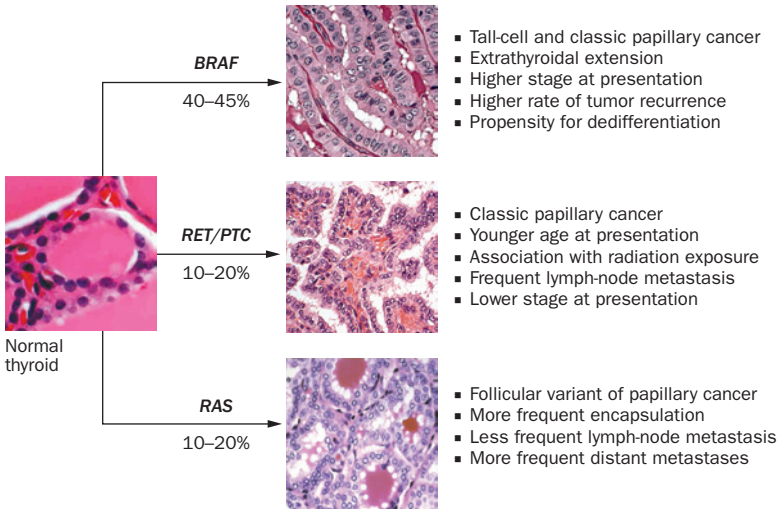

Figure 3 | Molecular alterations in papillary thyroid cancer and their average prevalence and association with clinical and histopathological features of tumors. Adapted from *Expert Reviews of Molecular Diagnostics* **8** (1), 83–89 (2008) with permission from Expert Reviews Ltd.

BRAF mutations

BRAF is a serine-threonine kinase that is translocated to the cell membrane after being bound and activated by RAS, which results in the phosphorylation and activation of MAPK kinase and other downstream targets of the MAPK signaling pathway. In thyroid cancer, BRAF can be activated by point mutations, small in-frame deletions or insertions or by chromosomal rearrangement. The most common mechanism of activation is a point mutation that involves a thymine to adenine substitution at nucleotide position 1799, which results in a valine-to-glutamate replacement at residue 600 (Val600Glu).[16,68] This *BRAF* mutation constitutes 98–99% of all *BRAF* mutations found in thyroid cancer. Other alterations include a Lys601Glu point mutation and small, in-frame insertions or deletions surrounding codon 600,[69–73] as well as *AKAP9/BRAF* rearrangement.[74] The rearrangement is a paracentric inversion of chromosome 7q that leads to the fusion between the portion of the *BRAF* gene that encodes the protein kinase domain and the *AKAP9* gene.[74] All point mutations and the rearrangement lead to the activation of BRAF kinase and chronic stimulation of the MAPK pathway.

The BRAF Val600Glu amino acid substitution is the most frequent genetic alteration in papillary thyroid cancer, being found in 40–45% of these tumors.[75] The mutation also occurs in 20–40% of poorly differentiated thyroid carcinomas and 30–40% of anaplastic thyroid carcinomas.[76–79] Many of these carcinomas also reveal areas of well-differentiated papillary cancer, and BRAF Val600Glu is present in both tumor components, which suggests that this mutation is an early event that predisposes the tumor to dedifferentiation. In papillary thyroid cancer, the BRAF Val600Glu substitution is typically found in tumors with classic papillary and tall-cell histology, and is rare in the follicular variant.[15,75] By contrast, tumors that harbor the *BRAF* mutation that causes the Lys601Glu amino acid substitution typically have the follicular variant of papillary carcinoma histology.[69,73]

PAX8/PPARγ rearrangement

This rearrangement leads to the fusion between a portion of the *PAX8* gene, which encodes a paired domain transcription factor, and the *PPARγ* gene.[80] The fusion results in strong overexpression of the chimeric PAX8/PPARγ protein,[80,81] although the mechanisms of its transforming activity remain to be fully understood.

PAX8/PPARγ is a prototypic alteration found in follicular thyroid carcinoma, where it occurs with a frequency of 30–35%.[82–84] In most studies, this rearrangement has also been found in some (2–13%) follicular adenomas and in a small proportion (1–5%) of the follicular variant of papillary carcinomas,[82–86] although occasional reports of a much higher prevalence of this rearrangement in the follicular variant of papillary carcinoma also exist.[87] *PAX8/PPARγ* rearrangements and *RAS* point mutations rarely overlap in the same tumor, which suggests that they represent distinct pathogenetic pathways in the development of follicular thyroid carcinomas.[83]

Mutations in tumor dedifferentiation

BRAF and *RAS* mutations are frequently found in both well-differentiated thyroid cancer and in poorly differentiated and anaplastic carcinomas and, therefore, probably represent an early event in thyroid cancer progression. Anaplastic and poorly differentiated carcinomas frequently have additional genetic alterations that are not found in well-differentiated cancers and, therefore, represent late events, which may be required to initiate tumor dedifferentiation. These late events include mutations of the *TP53* and *CTNNB1* genes, as well as mutation in genes that encode effectors of the PI3K–AKT signaling pathway.

Point mutations that affect the *TP53* gene (which encodes the cell cycle regulator p53) are found in 50–80% of cases of anaplastic carcinoma.[88–91] They are less frequently found in poorly differentiated carcinomas and are extremely rare in well-differentiated thyroid cancer. The mutations lead to loss of function of this important tumor suppressor gene. Another gene frequently mutated in anaplastic carcinoma is *CTNNB1*, which encodes a β-catenin that is involved in cell adhesion and Wnt signaling. Point mutations in exon 3 of the gene are found in up to 60% of anaplastic carcinomas, and these mutations also occur in poorly differentiated thyroid carcinomas, although with lower prevalence.[92–94]

Thyroid cancer dedifferentiation also involves progressive accumulation of other mutations, particularly those in genes that encode effectors of the PI3K–AKT pathway. Among anaplastic and poorly differentiated carcinomas, 10–20% of tumors harbor mutations in *PIK3CA* (the gene that encodes PI3K), 5–15% *PTEN* mutations and 5–10% *AKT1* mutations.[79,95–98]

Mutations in oncocytic tumors

Oncocytic tumors are characterized by cytoplasmic accumulation of innumerous mitochondria that frequently show abnormal morphology. The cause of the mitochondrial change and its relationship to the neoplastic process remain poorly understood. Mitochondrial abnormalities probably represent either a primary change associated with tumor initiation or a secondary change.[99–102]

Mutations of the gene *NDUFA13* (also known as *GRIM-19*) have been identified in oncocytic thyroid tumors.[103] This gene encodes a protein that regulates cell death and promotes apoptosis, and also affects mitochondrial metabolism by serving as an essential component of complex I of the mitochondrial respiratory chain.[104] In one study, somatic missense mutations in *NDUFA13* were found in 10–20% of oncocytic follicular carcinoma and the oncocytic variant of papillary carcinoma.[103] These mutations may disrupt the function of this antiapoptotic tumor suppressor gene and promote tumorigenesis. However, the role of *NDUFA13* mutations in carcinogenesis remains obscure.

Mutations in mitochondrial DNA are found with a high frequency in oncocytic carcinomas.[105,106] These mutations, which include deletions, frameshift and missense point mutations, affect genes that encode proteins of the mitochondrial complex I and other mitochondrial complexes that may lead to mitochondrial dysfunction.[99,107] Whether or not they have a causative role in tumor initiation remains unclear.

Other molecular events

Distinct alterations in gene expression have been observed in papillary carcinomas and other types of thyroid cancer.[18,108–111] These alterations include downregulation of genes responsible for specialized thyroid function (such as thyroid hormone synthesis), upregulation of many genes involved in cell adhesion, motility and cell–cell interaction, and different patterns of deregulation of the expression of genes that encode cytokines and other proteins involved in inflammation and immune response. Although the list of specific dysregulated genes varies substantially between different studies, a number of genes have reproducibly been found to be dysregulated at the mRNA level; they include *MET*, *TPO*, *TIMP1*, *DPP4*, *LGALS3* and *KRT19*.[108–110,112,113]

Among papillary carcinomas, different mRNA expression profiles have been observed in the classic papillary, follicular and tall-cell variants.[108,114] Moreover, significant correlations have been observed between *BRAF*, *RAS*, *RET/PTC* and *TRK* mutations and specific patterns of gene expression. This information has shed light on the molecular basis for the distinct phenotypic and biological features associated with each mutation type.[18,108] Acquisition of more invasive tumor characteristics and dedifferentiation of *BRAF*-mutated cancers seems to coincide with profound deregulation of the expression of genes that encode proteins involved in cell adhesion and the intercellular junction, which provides evidence for induction of an epithelial–mesenchymal transition—a process of switching from an epithelial to a mesenchymal phenotype that increases cell motility and invasiveness.[115,116]

miRNAs are small noncoding RNAs that function as negative regulators of protein-coding gene expression. Many miRNAs have been found to be deregulated in thyroid cancer.[117–120] Generally, miRNA expression profiles of papillary carcinoma are different from those of follicular carcinoma and other thyroid tumors.[121] Several specific miRNAs, such as miR-146b, miR-221 and miR-222, are highly upregulated in papillary carcinomas and may have a pathogenetic role in the development of these tumors.[118,120,121] Possible target genes of these miRNAs are the regulator of the cell cycle *p27(Kip1)* gene and the thyroid hormone receptor (*THRβ*) gene.[122,123] Several abnormally expressed miRNAs have also been found in follicular carcinomas (miR-197, miR-346, miR-155 and miR-224)[119,124] and anaplastic carcinomas (miR-30d, miR-125b, miR-26a and miR-30a-5p).[125]

Alteration in gene expression owing to aberrant methylation of gene promoter regions or histone modification also occurs in thyroid cancer. These epigenetic events can alter the function of tumor suppressor genes and thus contribute to activation of important signaling pathways, such as PI3K–AKT and MAPK cascades. Changes in epigenetic regulation might also result in downregulation of thyroid-specific genes during tumor progression and dedifferentiation.[126–128] Hypermethylation of the metalloproteinase inhibitor gene *TIMP3* and other tumor suppressor genes is frequently observed in thyroid cancers with the BRAF Val600Glu amino acid substitution, which may contribute to the aggressive biological behavior of tumors carrying this mutation.[129]

Mutation mechanisms and cancer etiology

A strong association exists between papillary thyroid cancer-specific chromosomal rearrangements and exposure to ionizing radiation, which is a well-known risk factor for thyroid cancer. *RET/PTC* rearrangements are found in up to 80% of papillary carcinomas in individuals exposed to either accidental radiation (mostly radioiodine) or therapeutic radiation (mostly external beam).[130–132] Radiation exposure after the Chernobyl accident particularly increased the frequency of the *RET/PTC3* rearrangement type and of novel types of *RET/PTC*.[130–133] Another chromosomal rearrangement, *BRAF/AKAP9*, was also found predominantly in papillary carcinomas associated with radiation exposure.[74] Among children exposed to radiation after the Chernobyl accident who developed thyroid cancer, the prevalence of both *RET/PTC* and *BRAF/AKAP9* was particularly high in tumors that manifested <10 years after the accident as compared to those that developed after a longer latency.[74,130] The opposite is true for point mutations involving the *BRAF* and *RAS* genes, which are rare in radiation-related tumors but common in the general population.[134] Among papillary carcinomas found in atomic bomb survivors in Japan (where doses received by the thyroid gland were calculated with high precision), a strong positive correlation was found between increased frequency of *RET/PTC* rearrangement and increased radiation dose, whereas frequency of *BRAF* point mutations showed an inverse correlation with the radiation dose.[135,136]

The role of radiation exposure in the generation of *RET/PTC* is also supported by the experimental induction of *RET/PTC* by irradiating human cultured thyroid cells[137,138] and fetal thyroid tissue xenografts in mice with severe combined immunodeficiency (SCID).[139,140] Although the exact molecular mechanisms involved in the formation of chromosomal rearrangements following radiation

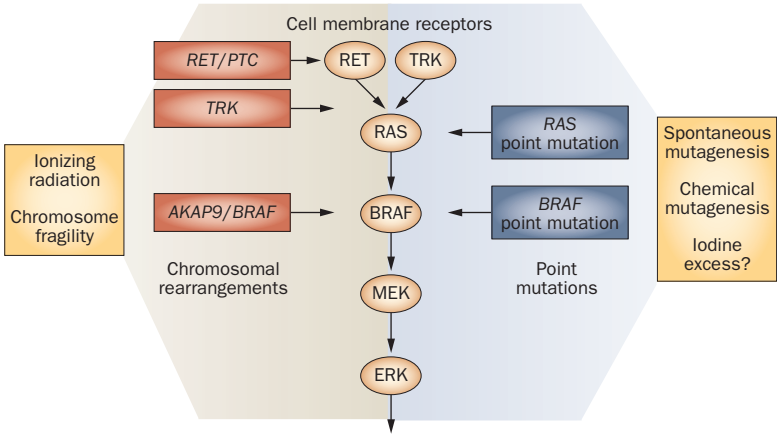

Figure 4 | In papillary thyroid cancer, activation of the MAPK signaling pathway occurs via two main mechanisms: chromosomal rearrangement or point mutations, which correlate with distinct etiologic factors. Chromosomal rearrangements have strong association with exposure to ionizing radiation and possibly with chromosome fragile sites. Point mutations lack the association with radiation exposure and probably develop as a result of chemical mutagenesis, with a possible influence of dietary iodine excess and exposure to certain chemical elements that are present in high concentrations in drinking water from volcanic areas. Permission obtained from the American Society for Clinical Investigation © Ciampi, R. *et al. J. Clin. Invest.* **115**, 94–101 (2005).

exposure are not well-understood, strong evidence exists that nuclear architecture contributes to the generation of *RET/PTC* and *TRK* by placing the potentially recombinogenic chromosomal loci in close proximity in the nuclei of normal human cells.[141–143] Spatial proximity probably predisposes the neighboring genes to simultaneous damage by radiation and facilitates mis-rejoining of free DNA ends located immediately adjacent to each other.

In addition to ionizing radiation, the induction of *RET/PTC* rearrangement may also be associated with chromosome fragility. The 10q11.2 and 10q21 regions on chromosome 10 where *RET* and *CCDC6*, its partner for the *RET/PTC1* rearrangement, reside have been known to contain fragile sites, FRA10G and FRA10C.[144,145] A study has mapped these fragile sites directly to the *RET* and *CCDC6* genes and demonstrated that induction of fragility at these fragile sites in cultured thyroid cells results in the generation of the *RET/PTC1* rearrangement.[146] Chromosome fragility may be caused or enhanced by hypoxia, ethanol, caffeine or other endogenous and exogenous factors;[144,145] therefore, it might represent another mechanism of formation of *RET/PTC* in thyroid cells. This mechanism would particularly be likely to occur in young individuals with papillary thyroid cancer, as in this setting *RET/PTC* rearrangements are common,[130,131,147] whereas the history of radiation is traceable only in the minority of patients.

In contrast to chromosomal rearrangements, point mutations are prevalent in thyroid cancers that lack the association with radiation exposure. Other etiologic factors must, therefore, be involved in their generation. A study has reported a higher prevalence of *BRAF* point mutations in papillary carcinomas from several regions of China with very high iodine contents in drinking water compared with regions with normal iodine content.[148] The observation was based on the analysis of >1,000 cases of classic papillary carcinomas and showed an odds ratio of 1.97 for BRAF Val600Glu association with high iodine levels. Although this study is far from being conclusive in establishing the causal association between high iodine intake and *BRAF* mutation, it points to this possibility. If confirmed, this finding would provide a biological basis for a significantly higher ratio of papillary to follicular thyroid cancer consistently observed in areas with high iodine intake compared with areas with moderate iodine intake or iodine deficiency.[149] Conceivably, the increase in the level of iodine intake may be one of the factors contributing to the growing incidence of papillary thyroid cancer. However, whether the worldwide increase in the incidence of papillary thyroid cancers coincides with the growing incidence of clinically relevant, *BRAF* mutation-positive cancers remains unknown.

Another study has reported a significantly higher incidence of *BRAF*-positive papillary carcinomas in a volcanic region of Sicily that has a higher concentration of boron, iron, vanadium, manganese and other chemicals in drinking water than neighboring regions.[150] The finding raises the possibility that mutagenesis leading to *BRAF* point mutations might be related to excessive exposure to specific chemical compounds found in volcanic areas. In support of this possibility, the Hawaiian islands belong to the region of the world with the highest incidence of thyroid cancer, and they host multiple volcanoes, many of which are active.[151] However, the prevalence of *BRAF* mutations in papillary carcinomas from these areas has not been reported.

In summary, the available data provide evidence that in papillary thyroid cancer, distinct mutational mechanisms are linked to specific environmental factors that have a role in thyroid carcinogenesis (Figure 4).

Clinical utility of molecular markers

Mutational markers

Molecular markers hold great promise in improving the diagnosis of cancer in patients with thyroid nodules. Such improvement would particularly benefit patients with nodules that are classified as indeterminate for malignancy by FNA cytology. The inability to rule out cancer in these nodules leads to diagnostic lobectomy for most of these patients, although 60–90% of the surgically removed thyroid nodules are found to be benign.[152–154] Those patients that are found to have cancer in their nodules after initial surgery are also treated suboptimally, as they have to undergo a second surgery to complete the thyroidectomy. Both the unnecessary surgeries and the two-step surgical management can be avoided with more accurate preoperative diagnosis of cancer in thyroid nodules.

Diagnostic use of mutational markers for the analysis of thyroid FNA samples has been explored for single genes and for a panel of mutations. Among single genes, the majority of studies have focused on *BRAF* mutations. A meta-analysis of the results reported in 22 studies of thyroid FNA samples tested for *BRAF* mutation[155–158] revealed that out of 1,117 nodules found to be positive for the Val600Glu substitution, 1,109 (99.3%) were classified as papillary carcinomas on final histopathology. Eight cases

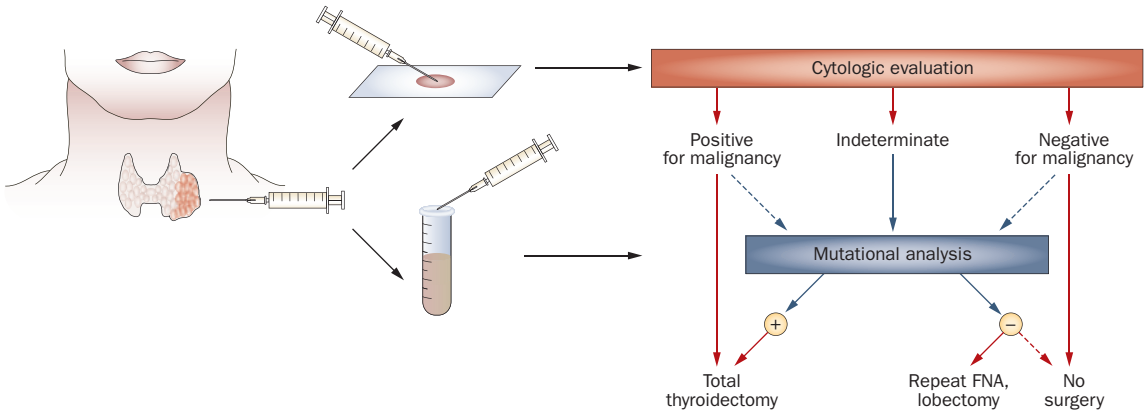

Figure 5 | Potential clinical management of patients with thyroid nodules on the basis of a combination of cytological examination and molecular analysis. Molecular testing can be particularly helpful for nodules with indeterminate cytology. Nodules positive for mutations indicate a high risk of cancer; therefore, patients with these nodules can be treated by total thyroidectomy. Patients with nodules that yield an indeterminate diagnosis on cytology but are negative for mutations might require a repeated FNA and diagnostic lobectomy, although consideration of following up some of these patients annually may be given, particularly for patients with the cytologic diagnosis of atypia of undetermined significance/follicular lesion of undetermined significance. Molecular testing of nodules found to be negative for malignancy by cytology decreases the rate of false-negative cytologic results, but its cost-effectiveness has not been assessed. Molecular testing of samples classified as malignant by cytology can identify *BRAF*-positive tumors, which may require more extensive surgery than *BRAF*-negative tumors, although specific recommendations for surgical management of thyroid cancer based on the mutational status have not been developed yet. Abbreviation: FNA, fine-needle aspiration.

were false-positive, as no cancer was found after surgery, of which five cases were reported in one study that used ultrasensitive detection of this mutation.[157] Even if these cases are accepted as true false-positive, *BRAF* mutation appears to be a highly accurate marker of cancer in thyroid nodules sampled by FNA and confers a risk of malignancy of >99%. Importantly, 15–40% of the samples that were positive for the *BRAF* mutation had indeterminate FNA cytology, which indicates that *BRAF* can be of considerable diagnostic value in the evaluation of these nodules.[156,157,159–164]

Despite high specificity for cancer, testing for *BRAF* mutation alone misses many thyroid cancers that are negative for this mutation. The performance of molecular testing can be improved by including other frequently occurring mutations in the analysis. Use of a panel of mutations including *BRAF* and *RAS* point mutations and *RET/PTC* and *PAX8/PPARγ* rearrangements, with the possible addition of the *TRK* rearrangement, for analysis of thyroid FNA samples has been explored.[159,165–167] Studies that evaluated the use of this panel in a setting of the clinical diagnostic laboratory demonstrated that finding any mutation was a strong predictor of malignancy in thyroid nodules irrespective of the cytological diagnosis.[159,165,166] In these studies, the presence of *BRAF*, *RET/PTC* or *PAX8/PPARγ* correlated with the malignant outcome in 100% of cases, whereas *RAS* mutations had a 74–87% positive predictive value for cancer. On the basis of the high probability of cancer in nodules positive for mutations, these patients, with the possible exception of patients with *RAS*-positive nodules, can be treated by total thyroidectomy as the initial surgical approach (Figure 5).

Other FNA-based studies have reported a lower specificity of mutations for cancer, particularly of *RET/PTC* rearrangement.[167] The decrease in specificity of *RET/PTC* detection for cancer in thyroid nodules is proportional to the increase in sensitivity of the detection techniques.[49,168] If the panel of mutations is intended for clinical diagnostic use, therefore, the sensitivity of the detection of all mutations should be first validated using a set of retrospective samples obtained from confirmed malignant and benign thyroid nodules.

The use of the appropriately validated analysis for a panel of mutations could be particularly useful for the assessment of nodules assigned to the lowest cancer risk category of indeterminate cytology, namely, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS).[154,169] Patients with the cytological diagnosis of AUS/FLUS have a 5–15% probability of cancer and typically undergo repeated FNA biopsies and, if the diagnosis remains the same, are treated by thyroid lobectomy.[154,169] The most common mutation found in these nodules is in the *RAS* gene, followed by mutations in *BRAF* and the *PAX8/PPARγ* rearrangement. Detection of these mutations in nodules with AUS/FLUS cytology confers a high risk of cancer,[159,166] which may enable many patients to bypass repeated FNA biopsies and proceed to optimal surgical management without delay. The risk of cancer in nodules with AUS/FLUS cytology that are negative for all mutations is approximately 7%.[159,166] Whether this cancer risk is sufficiently low to follow up these patients without surgery is unclear. The accumulation of knowledge on the diagnostic use of molecular markers has been reflected in the revised American Thyroid Association's management guidelines, which recommend clinicians to consider the use of a mutational panel for nodules with indeterminate FNA cytology to help guide clinical management.[170]

The potential role of testing FNA samples deemed negative for malignancy for mutations might be in decreasing the rate of false-negative cytology. Molecular testing

decreased the rate of false-negative cytology from 2.1% to 0.9% in one study[159] and enabled the detection of six out of nine cancers in 87 nodules with benign cytology in another observation.[165] However, the cost-effectiveness of molecular testing of all negative cytology samples requires further investigation.

Similarly, it remains to be established if molecular testing of FNA samples found to be positive for malignancy on cytology should be performed and taken into account in the surgical and postsurgical management of patients with thyroid cancer. BRAF Val600Glu has been associated in many studies with aggressive histopathologic features of papillary carcinoma, including extrathyroidal extension, lymph-node metastases and more advance stage at presentation.[171] Moreover, an association between BRAF Val600Glu and tumor recurrence[172–175] and tumor-related mortality[176,177] has been shown. Tumors carrying this mutation more frequently require reoperation for locally persistent or recurrent disease than tumors without the mutation.[177,178] These findings suggest that patients with BRAF Val600Glu-positive papillary thyroid cancers detected preoperatively may benefit from more extensive initial surgery.

Furthermore, patients with BRAF Val600Glu-positive tumors have an increased chance of failing to respond to radioiodine treatment of recurrent disease. The decreased response to radioiodine treatment is probably due to alteration of function of the sodium iodide symporter (NIS) and other proteins that metabolize iodide in cells harboring this mutation, which thereby decreases the ability of these tumors to trap radioiodine.[79,172,179,180] An increased dose of radioiodine has, therefore, been suggested for initial postoperative treatment of patients with *BRAF*-positive cancer.[181] It has also been proposed that these patients might benefit from lower levels of suppression of TSH and closer postsurgical follow-up.[181]

The association between BRAF Val600Glu and aggressive disease characteristics has also been reported in small, early-stage tumors[73,172] and in thyroid papillary microcarcinomas, which are often incidentally discovered tumors of 1 cm or less in size.[182–187] These findings suggest that BRAF Val600Glu could potentially be used for risk stratification and individualized clinical management of patients with papillary thyroid cancer. However, the use of *BRAF* alone for risk stratification of thyroid papillary carcinomas and microcarcinomas remains controversial,[188] particularly because the BRAF Val600Glu mutation is found in ~45% of papillary carcinomas, whereas less than 10–15% of these tumors are known to have aggressive clinical behavior. Therefore, it is probable that not all tumors harboring BRAF Val600Glu have potential for worse outcome, and additional factors exist that can modify the outcome of patients with tumors positive for this mutation. Indeed, a study has suggested that the association between *BRAF* mutations and increased risk of tumor recurrence is limited to older (≥65 years) patients.[189] The results of another study suggest that risk stratification of patients with thyroid microcarcinomas could involve the combination of *BRAF* and several specific histopathologic tumor features.[190] *BRAF* will probably, therefore, be used in combination with other clinical or histopathological parameters to accurately define a subset of tumors with potentially unfavorable outcomes.

Other markers

In addition to gene mutations, expression of mRNA and miRNA markers has also been explored for the diagnostic assessment of thyroid FNA samples. Use of a limited number of differentially expressed gene markers shows promise, although no reliable single marker or reproducible subset of markers has been discovered yet. Upregulation of the *HMGA2* gene in malignant thyroid tumors has been found in several studies and might be of some diagnostic use in assessment of FNA samples.[113,191–194] Aberrant expression of *MET*, *TPO*, *TIMP1*, *DPP4* and other genes has been observed in several studies and explored for diagnostic use.[108–110,112,113] One study has tested the possibility of using expression levels of multiple genes for cancer detection in thyroid FNA samples.[195] By applying a commercially developed algorithm to a set of 48 thyroid FNA samples, the researchers were able to distinguish benign from malignant nodules with a sensitivity of 92% and specificity of 84%. The results of this study, although performed using a small set of samples, are promising. The utility of this algorithm for diagnostic assessment of FNA samples is pending validation in a larger set of thyroid FNA samples and in other, non-industry-sponsored studies. The possibility of combining the use of cytological evaluation, mutational analysis and gene expression markers to improve the diagnostic assessment of FNA samples from thyroid nodules has also been investigated.[194] A study has suggested that the use of *NRAS* mutation status combined with the analysis of *TIMP1* expression may further improve the diagnostic accuracy of FNA cytology.

Several miRNAs, including miR-221, miR-222 and miR-146b, are consistently overexpressed in papillary thyroid carcinomas and could be of potential diagnostic use.[117,120,196] The diagnostic utility of measuring the expression level of miRNAs in thyroid FNA samples has been explored using a panel of seven miRNAs (miR-187, miR-221, miR-222, miR-146b, miR-224, miR-155 and miR-197).[119] Upregulation of three or more of these miRNAs could predict papillary or follicular thyroid cancer in an FNA sample with the accuracy of 98%. This result demonstrates the potential feasibility of miRNA analysis in thyroid FNA samples and provides initial evidence for its possible diagnostic use. However, the clinical utility of miRNA markers requires further validation.

miRNAs have also been explored as markers of aggressive behavior of papillary thyroid carcinoma. Upregulation of miR-146b and miR-222 and downregulation of miR-34b and miR-130b have been found to be associated with aggressive behavior of papillary thyroid cancer in one study,[197] whereas another observation showed that high levels of miR-221, miR-222 and miR-146b expression correlated with extrathyroidal invasion in papillary carcinoma.[198]

Conclusions

Progress in our understanding of the molecular genetics of thyroid cancer will probably accelerate in the near future

owing to the increasing availability of array-type and next-generation sequencing technologies. This knowledge should lead to the discovery of novel mutations and other genetic and epigenetic events in thyroid cancer.

The association between chromosomal rearrangements and radiation exposure is well-established; however, the precise mechanisms of gene recombination following DNA damage induced by ionizing radiation remains to be uncovered. Further studies are also needed to unravel the etiologic factors leading to point mutations in thyroid tumors, particularly to better understand the role of dietary iodine and other environmental factors in the formation of *BRAF* point mutations. These findings could shed light on the increasing incidence of thyroid cancer observed in many countries during the past 20–30 years.

Finally, the accumulation of knowledge in molecular genetics of thyroid cancer has begun to affect the clinical management of patients and is poised to dramatically improve the accuracy of cancer detection in thyroid nodules, cancer prognostication and selection of therapeutic targets for molecular therapy of advanced thyroid cancer. Substantial progress has already been achieved in improving the diagnosis of cancer in nodules with indeterminate cytology. We believe that further advances in thyroid cancer biology will eliminate this uncertain diagnostic category, which will have a profound effect on clinical management of patients with thyroid nodules.

Review criteria

A search for full-text articles in the English language published between 1980 and 2011 was performed in PubMed. The keywords used included "thyroid cancer" alone and in combination with "molecular genetics", "BRAF", "RET/PTC", "RAS", "PAX8/PPARgamma", "FNA diagnosis", and "radiation exposure". Reference lists in selected articles were used to further expand the search. Whenever appropriate, more recent references or studies that employed the largest series of patients were used.

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Author contributions
Both authors contributed equally to all aspects of the article.