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Molecular analysis of thyroid tumors

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In the recent years, a large number of molecular alterations in thyroid cancer has been discovered and characterized. Some of these markers may have significant diagnostic utility, can be used for tumor prognostication, and serve as potential therapeutic targets. The diagnostic utility of these markers is of particular importance in thyroid fine-needle aspiration samples. Some molecular markers, such as BRAF, offer help in risk stratification and can be potentially used to optimize surgical and postsurgical management of patients with thyroid cancer. This review discusses major molecular alterations known to occur in thyroid cancer, focusing on those markers that have been extensively characterized, carry clinical significance, and are being introduced into pathology practice.
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In the recent years, our understanding of molecular genetics of thyroid cancer has expanded dramatically. This knowledge contributes to better understanding of thyroid tumor biology and has started to translate into clinical practice as an ancillary tool for cytological and pathological diagnosis of thyroid cancer and for better tumor prognostication.

Molecular alterations in thyroid cancer

Four mutation types, that is, *BRAF* and *RAS* point mutations and *RET/PTC* and *PAX8/*peroxisome proliferator-activated receptor γ (*PPAR*γ) rearrangements, constitute the majority of mutations known to occur in the two most common types of thyroid cancer, papillary and follicular carcinoma. At this time, they carry the highest impact on tumor diagnosis and prognostication. Papillary carcinomas harbor point mutations of the *BRAF* and *RAS* genes and *RET/PTC* rearrangements, all of which are able to activate the mitogen-activated protein kinase (MAPK) pathway. These mutually exclusive mutations are found in >70% of papillary thyroid carcinomas.[1–4] Follicular carcinomas are known to harbor either *RAS* mutations or *PAX8/PPAR*γ rearrangement. These mutations are also mutually exclusive and identified in ~75% of follicular carcinomas.[5]

Another type of genetic alterations in thyroid cancer involve the PI3K/AKT signaling pathway, although they are rare in well-differentiated thyroid cancer and have higher prevalence in less-differentiated thyroid carcinomas.[6–8] Additional mutations known to occur in poorly differentiated and anaplastic carcinomas involve the *TP53* and *CTNNB1* genes.[9] However, these mutations are rare in well-differentiated papillary or follicular carcinoma. *TRK* rearrangement represents another type of chromosomal rearrangement that occurs in papillary thyroid carcinomas,[10–12] although its prevalence is low, particularly in North America (<5%).

BRAF

Mutations of the *BRAF* gene have been reported in 35–70% of papillary thyroid carcinomas, although in most studies the prevalence in close to 40–45%, making it the most common known genetic event in papillary thyroid carcinomas.[2,13,14] The vast majority (>95%) of *BRAF* mutations found in thyroid cancer are a thymine to adenine transversion at nucleotide 1799 (T1799A) leading to a substitution of valine by glutamic acid at residue 600 of the protein (V600E). This point mutation leads to constitutive activation of BRAF kinase and chronic stimulation of the MAPK pathway, and is tumorigenic for thyroid cells. Other and rare mechanisms of BRAF activation in thyroid papillary cancer include K601E point mutation, small in-frame insertions or deletions

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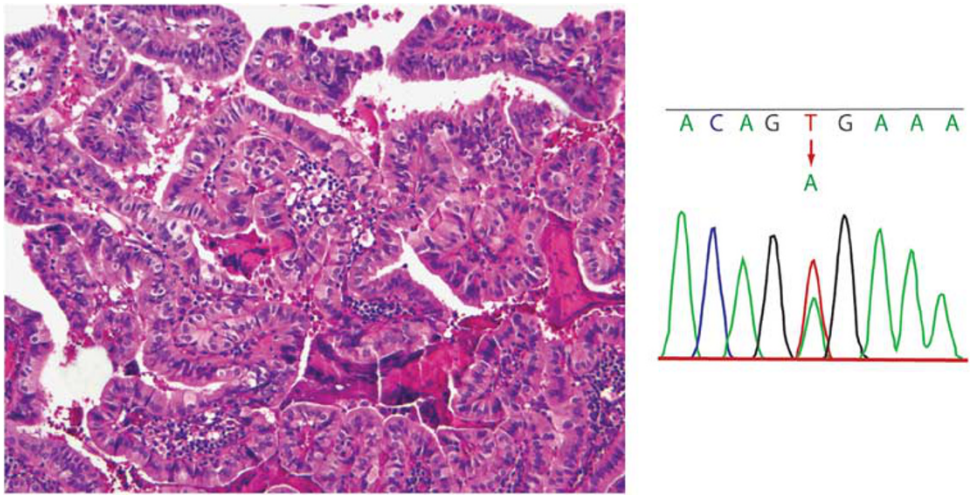

Figure 1 *Left*: Microscopic appearance of the tall cell variant papillary carcinoma. *Right*: The tumor was positive for *BRAF* codon 600 GTG→GAG mutation leading to V600E, as detected by Sanger sequencing.

surrounding codon 600,[15] and *AKAP9/BRAF* rearrangement, which is more common in papillary carcinomas associated with radiation exposure.[16]

BRAF V600E mutation has a strong association with specific histological variants of papillary carcinoma. It is highly prevalent in the tall cell variant of papillary carcinoma (Figure 1), where it occurs in 70–80% of cases, and in tumors with classic papillary growth (~60%).[1,13,17] Many BRAF-positive tumors are classic papillary carcinomas with focally prominent tall cell features or diffuse borderline tall cell features. BRAF-positive classic papillary carcinomas typically have an invasive border and may show focal areas composed of tumor cells with tall cell features. On the contrary, *BRAF* V600E is found only in about 10% of follicular variants of papillary carcinoma. Another *BRAF* point mutation, K601E, which is overall rare, is found mostly in the follicular variant of papillary carcinoma.[18,19] *BRAF* V600E mutation can also be seen in anaplastic and poorly differentiated thyroid carcinomas arising from papillary carcinoma.[17,20,21] However, *BRAF* V600E is not found in follicular carcinomas and benign thyroid nodules, and therefore among primary thyroid lesions it represents a specific marker of papillary carcinoma and related tumor types.

In addition to higher incidence in the tall cell variant of papillary carcinoma, *BRAF* V600E mutation has also been shown to correlate with other aggressive characteristics of papillary carcinomas, including extrathyroidal extension, advanced tumor stage at presentation, and lymph node or distant metastases.[17,20,22,23]

RET/PTC

RET/PTC rearrangement is another genetic alteration found in papillary carcinomas.[24] It is formed by the fusion between the 3′ portion of the RET receptor tyrosine kinase gene and the 5′ portion of various unrelated genes. Two most common rearrangement types, *RET/PTC1* and *RET/PTC3*, are intrachromosomal inversions as both *RET* and its respective fusion partner genes, *H4* and *NCOA4* (also known as *ELE1*), are located on chromosome 10.[25–27] *RET/PTC2* and nine more recently identified types of *RET/PTC* are all interchromosomal translocations (reviewed in Ciampi *et al*[28]). All rearrangement types contain the intact tyrosine kinase domain of the *RET* receptor and enables the RET/PTC chimeric protein to activate the RAS–RAF–MAPK cascade and initiate thyroid tumorigenesis.

RET/PTC rearrangements are found on average in 10–20% of adult sporadic papillary carcinomas, although its prevalence is highly variable between various observations, largely because of the difference in sensitivity of the detection methods and also because of some geographical variability.[29,30] *RET/PTC* occur with higher incidence in patients with the history of radiation exposure (50–80%) and in papillary carcinomas from children and young adults (40–70%).[31–34] The distribution of *RET/PTC* rearrangement within tumor cells can vary from involving most of neoplastic cells (clonal *RET/PTC*) to being detected only in a small fraction (<1%) of tumor cells (nonclonal *RET/PTC*).[35,36] Although *RET/PTC* has been found in several studies in adenomas and other benign thyroid lesions, it can be assumed that clonal *RET/PTC* (ie, rearrangement that is found in most cells within the tumor) is reasonably specific for papillary thyroid carcinoma.[29,35] Two groups have reported the occurrence of *RET/PTC* in hyalinizing trabecular tumors.[37–39] In one observation, four tumors showed RET expression by immunohistochemistry and three of those were found to harbor *RET/PTC1* rearrangement by RT-PCR.[37] In another study, *RET/PTC1* was detected in six out of eight hyalinizing trabecular adenomas by RT-PCR.[38] These findings provide

evidence suggesting that hyalinizing trabecular tumors represent a peculiar variant of papillary carcinoma. However, these studies did not investigate whether or not *RET/PTC* was present in the majority of cells within these tumors and therefore cannot provide conclusive biological evidence for linkage between papillary carcinoma and hyalinizing trabecular tumor.

In papillary thyroid carcinomas, *RET/PTC1* is the most common and comprises 60–70% of all rearrangement types, whereas RET/*PTC3* accounts for 20–30% and *RET/PTC2* and other novel rearrangement types for <5%.[11,40] *RET/PTC*-positive papillary carcinomas, particularly those carrying *RET/PTC1* rearrangement, are typically present at younger age and have a high rate of lymph node metastases.[1] Most *RET/PTC*-positive tumors, particularly those harboring *RET/PTC1*, show classic papillary growth pattern, and *RET/PTC1* appears to be more common in papillary microcarcinomas.[40–42] The follicular variant of papillary carcinoma shows low prevalence of *RET/PTC*.[34,40] Among papillary carcinomas in children exposed to radiation after the Chernobyl accident, a strong correlation between different *RET/PTC* types and morphological variants of papillary carcinoma has been observed. In these populations, the solid variant of papillary carcinoma was associated with *RET/PTC3* and classic papillary carcinoma with *RET/PTC1*.[31,32,43] However, it remains unclear whether such phenotype–genotype correlation exists in the general population. It has not been found in a study of sporadic solid variant and classic papillary carcinomas, although the number of cases analyzed for *RET/PTC* in this study was small.[44]

RAS

Point mutations of the *RAS* gene are not restricted to a particular type of thyroid tumors and found in follicular carcinomas, papillary carcinomas, and follicular adenomas. The three human *RAS* genes (*HRAS*, *KRAS*, and *NRAS*) encode highly related G-proteins that propagate signals arising from cell membrane receptors to various intracellular targets. Point mutations in the specific domains of the *RAS* gene either increase its affinity for GTP (mutations in codons 12 and 13) or inactivate its autocatalytic GTPase function (mutation in codon 61), resulting in permanent RAS activation and chronic stimulation of its downstream targets along the MAPK and PI3K/AKT signaling pathways.

In thyroid tumors, mutations involving *NRAS* codon 61 and *HRAS* codon 61 are by far the most common, although mutations have been found in different hotspots of all three genes. In papillary carcinomas, *RAS* mutations occur in 15–20% of tumors.[45–50] Papillary carcinomas harboring *RAS* mutation almost always have the follicular variant histology (Figure 2); this mutation also correlates with significantly less prominent nuclear features of papillary carcinoma, more frequent encapsulation, and low rate of lymph node metastases.[1,51] *RAS* mutations are also found in 40–50% of conventional type follicular carcinomas[48,52–56] and 20–40% of conventional type follicular adenomas.[45,52–55] In adenomas, the mutations appeared to be more common in tumors with a microfollicular growth pattern.[52] A lower incidence has been reported in oncocytic tumors, in which only 0–4% of adenomas and 15–25% of carcinomas appeared to be affected.[54,57,58] *RAS* mutations have also been detected in few cold adenomatous nodules and goiter nodules,[45,54,59] although it is likely that these lesions are true neoplasms and therefore should be designated as follicular adenomas, despite their frequent macrofollicular colloid-rich histology.

PAX8/PPARγ

PAX8/PPARγ rearrangement is a result of translocation between chromosomes 2 and 3, t(2;3)(q13;p25), leading to the fusion between the *PAX8* gene coding

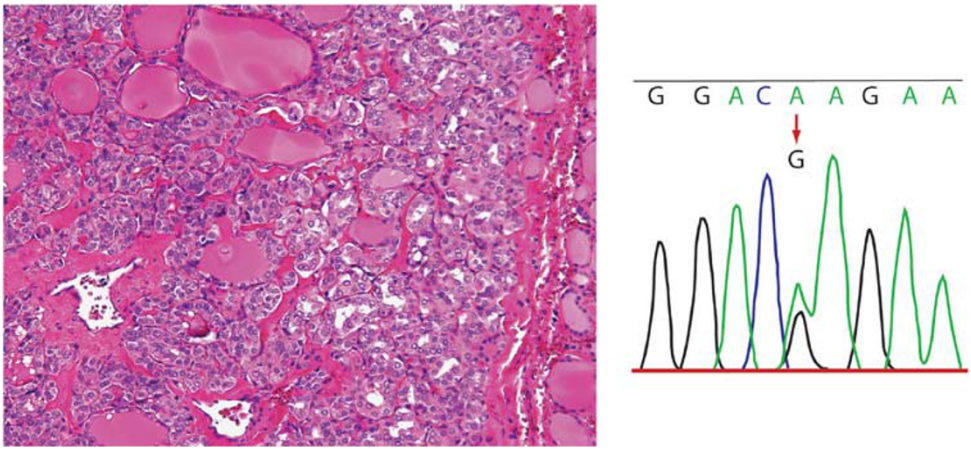

Figure 2 *Left*: Microscopic appearance of the encapsulated follicular variant of papillary carcinoma. *Right*: The tumor was positive for *NRAS* codon 61 CAA→CGA mutation leading to Q61R, as detected by Sanger sequencing.

for the thyroid-specific paired domain transcription factor, and the *PPARγ* gene.[60] *PAX8/PPARγ* rearrangement leads to strong overexpression of the PPARγ protein, although the mechanisms of cell transformation induced by this genetic event are yet to be fully understood.

PAX8/PPARγ is found in 30–40% of conventional type follicular carcinomas, and with lower prevalence in oncocytic carcinomas.[5,61,62] Tumors harboring *PAX8/PPARγ* tend to present at a younger age, are smaller in size, have a solid/nested growth pattern, and more frequently reveal vascular invasion.[5,19,61] This rearrangement can also be found in a small fraction (2–10%) of follicular adenomas and in some (<5%) follicular variant papillary carcinomas.[5,19,62,63] Follicular adenomas positive for *PAX8/PPARγ* typically have a thick capsule and show the immunohistochemical profile characteristic of thyroid cancer, suggesting that they may represent preinvasive (*in situ*) follicular carcinomas or malignant tumors in which invasion was overlooked during histological examination.[5] Single studies have reported a significantly higher frequency of *PAX8/PPARγ* rearrangement in follicular adenomas and follicular variant papillary carcinomas.[19] Another type of the *PPARγ* gene fusion, *CREB3L2/PPARγ*, has been reported in 1 out of 42 follicular carcinomas.[64]

Sample requirements and techniques for molecular analysis

Modern molecular techniques allow the detection of various genetic alterations in fine-needle aspiration (FNA) samples and in various types of surgically removed tumor tissues. A snap-frozen tumor sample generally represents the best source of material for molecular testing. If the fixed material has to be used, the formalin fixation or cytological ethanol fixation should be used. Tissue exposed to decalcifying solution, B5, and most of other special fixatives degrade nucleic acids and therefore cannot be used for molecular analysis. The choice of most appropriate detection technique is determined by the mutation type and samples type available for the analysis.

Testing for point mutations, such as those of *BRAF* and *RAS*, is relatively straightforward and can be reliably performed in freshly frozen and fixed samples. A variety of available molecular techniques can be used, including conventional PCR and Sanger sequencing, pyrosequencing, real-time PCR amplification and post-PCR melting curve analysis, allele-specific PCR, and others.[2,17,65–70] These techniques typically provide a reliable and sensitive detection of *BRAF* mutations in various types of thyroid samples. One study that compared four different approaches, that is, probe-specific real-time PCR, real-time allele-specific PCR, direct sequencing, and colorimetric assay, found all the techniques showing similarly high sensitivity in the detection of *BRAF* mutation in fixed FNA samples.[66]

The choice of a method for the detection of chromosomal rearrangements, such as *RET/PTC* and *PAX8/PPARγ*, is dictated largely by the type of sample available. When freshly collected or snap-frozen FNA or tumor tissue samples are available, the testing can be reliably performed by RT-PCR. The sensitivity of detection should generally be not higher than 1% of tumor cells (ie, should detect 1% or more tumors cells in the background of normal cells) to avoid detecting nonclonal rearrangements, which have no diagnostic implications. When only formalin-fixed and paraffin-embedded tissue is available for testing, fluorescence *in situ* hybridization is the assay of choice. The appropriate cutoff levels have to be established, which generally should be no <8–12% of cells with the rearrangement pattern of signals, as this assure the reliable detection and avoid detecting nonclonal rearrangements.[71] Usage of RT-PCR for clinical detection of *RET/PTC* and *PAX8/PPARγ* rearrangements in formalin-fixed and paraffin-embedded tissues should be avoided due to severe RNA degradation, which can not be compensated by choosing ultrasensitive conditions. The latter results in the loss of diagnostic specificity of the test due to increased detection of nonclonal rearrangement and higher risk of false-positive results.

Role of molecular markers in thyroid cancer diagnosis

Molecular Analysis of FNA Samples

FNA cytology is currently the most reliable diagnostic test for thyroid nodules and establishes the definitive diagnosis of a benign or malignant lesion in the majority of cases, whereas 10–40% of all FNA samples are diagnosed as indeterminate for malignancy.[72–75] The general category of indeterminate cytology encompasses several subcategories, that is, follicular lesion of indeterminate significance (FLUS), follicular neoplasm/Hürthle cell neoplasm, and suspicious for malignancy, which correlate with the estimated risk of malignancy of 5–10, 20–30, and 50–75%, respectively.[76] Owing to the lack of definitive diagnosis, most patients with indeterminate cytology undergo surgery, although only 8–17% of surgically removed thyroid nodules are malignant.[77,78] Patients with indeterminate FNA cytology and malignant tumors are not adequately treated as well, as most of them initially undergo thyroid lobectomy and later have another surgery to complete thyroidectomy.

Molecular testing of FNA samples may significantly improve the accuracy of cytological diagnosis of thyroid nodules. Most experience to date is accumulated for *BRAF* mutations. The results of *BRAF* testing in 2766 FNA samples have been reported in 18 prospective and retrospective studies.[65,66,69,70,79–92] Among 581 *BRAF*-positive nodules tested in FNA samples in these studies,

580 were papillary carcinomas on pathological examination of the resected nodules, whereas one was diagnosed as a benign nodule,[92] resulting in the false-positive rate of 0.2%. This reportedly benign nodule had a histopathological diagnosis of 'atypical nodular hyperplasia' and was not examined using modern immunohistochemical techniques that are helpful in the diagnosis of thyroid malignancy in difficult cases.[93] Importantly, 15–40% of *BRAF*-positive FNA samples are indeterminate or nondiagnostic by cytology,[69,70,82,85,87,88] indicating that testing for *BRAF* is helpful in establishing the definitive diagnosis of cancer in nodules with indeterminate cytology.

In addition to *BRAF*, several studies have explored the possibility of the detection of *RET/PTC*, *TRK*, or *RAS* mutations in thyroid FNA samples.[88,94,95] However, the biggest diagnostic impact can be achieved by testing FNA samples for a panel of mutations rather then for a single mutation. Recent studies have explored the diagnostic utility of molecular testing for a panel of mutations consisting of *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARγ*.[69,96] One study examined 470 consecutive FNA samples from thyroid nodules that were prospectively tested and yielded 32 mutations.[69] The presence of any mutation was a strong predictor of cancer, as 31 (97%) of mutation-positive nodules had a malignant diagnosis after surgery and one case (3%) was a follicular adenoma. This study showed that testing for a panel of mutations was particularly useful in nodules with indeterminate cytology, especially in the lowest risk subgroup of indeterminate cytology, that is, FLUS. In this subgroup, the positive mutational status had a 100% accuracy in predicting the risk of a malignancy, whereas mutation-negative nodules were all benign. In addition, this study showed that molecular testing decreased the false-negative rate of cytology from 2.1 to 0.9%.[69] Another study focused only on the FLUS group of cytology samples and found that testing for mutations had a 100% positive predictive value and 92% negative predictive value for cancer in this group (Figure 3).[96] Regarding specific mutations that constituted the panel, *BRAF*, *RET/PTC*, and *PAX8/PPARγ* mutations had a 100% positive predictive value for cancer in both studies.[69,96] Detection of *RAS* mutation, which was the second most common mutation after *BRAF*, also appeared to be of high diagnostic value in FNA samples, as it conferred an 87–100% probability of malignancy. Importantly, *RAS* mutations were identified in tumors, which are difficult to diagnose by cytology alone, that is, follicular variant of papillary carcinoma and follicular carcinoma. The accumulation of knowledge on diagnostic use of molecular markers has been reflected in the Revised Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer, recently released by the American Thyroid Association.[97] The guidelines recommend the use of molecular markers, such as *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARγ*, for indeterminate FNA cytology to help guide patient management.

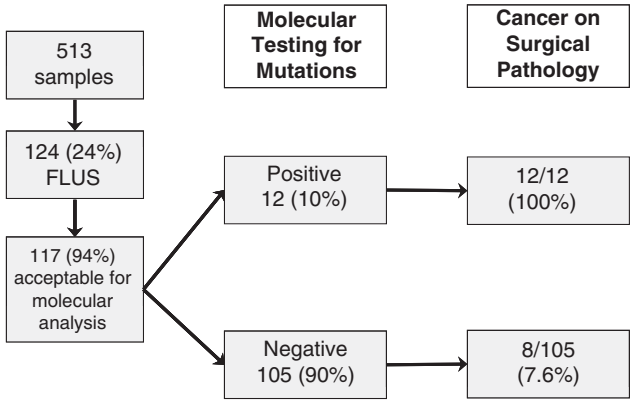

Figure 3 Diagnostic significance of mutational testing in thyroid FNA samples with FLUS cytology. On the basis of the results reported by Ohori *et al*.[96]

Molecular Analysis of Surgically Removed Tumors

Molecular testing of surgically removed thyroid samples has a rather limited diagnostic impact in papillary carcinomas. When the histopathological diagnosis of papillary carcinoma is suspected, testing of tumor tissue for *BRAF* and clonal *RET/PTC* can be of diagnostic value as their presence is a strong indicator of papillary carcinoma. However, many tumors carrying these mutations are classic papillary carcinomas or tall cell variants, and in those cases the histological diagnosis is typically straightforward. The follicular variant of papillary carcinoma, which more often imposes diagnostic difficulty on surgical pathology examination, most frequently has *RAS* mutations. However, *RAS* mutation cannot be used to define malignancy as it can be found in follicular adenomas.

In follicular carcinomas, testing for *PAX8/PPARγ* rearrangement may be of significant diagnostic value. This mutation is characteristically seen in follicular carcinomas, although, as discussed earlier, it can also be seen in a small fraction of follicular adenomas. The finding of *PAX8/PPARγ* should prompt the pathologist to perform an exhaustive search for vascular or capsular invasion. The invasion may not be seen at first, but is detected in many *PAX8/PPARγ*-positive follicular tumors after examination of the entire tumor capsule in multiple histological levels.[5,61,98]

Molecular markers of aggressiveness in thyroid cancer

BRAF

BRAF V600E mutation is generally considered as a reliable prognostic marker for papillary carcinoma.

Its association with more unfavorable tumor behavior has been documented in most studies involving sufficiently large cohorts of patients, although some observations have not found such an association (reviewed in Xing[23]). In many studies, *BRAF* V600E mutation correlate with aggressive tumor characteristics, such as tall cell variant, extrathyroidal extension, advanced tumor stage at presentation, and lymph node or distant metastases (reviewed in Xing[23]). More importantly, *BRAF* V600E has been found to be an independent predictor of treatment failure and tumor recurrence, even in patients with low-stage disease.[22,99,100] A recent study of 102 papillary carcinomas with median follow-up of 15 years demonstrated that *BRAF* V600E mutation was an independent risk factor for tumor-related death.[101] *BRAF* V600E association with disease-free probability remains significant when mutation was detected not only in surgically removed tumor samples but also in thyroid FNA samples.[90]

BRAF activation via *BRAF* V600E mutation in thyroid cells appears to lead to the alteration of function of sodium iodide symporter (*NIS*) and other genes metabolizing iodide, which is likely to be responsible for the decreased ability of tumors with *BRAF* mutation to trap radioiodine and treatment failure of the recurrent disease.[8,102,103] *BRAF* mutation also predisposes to tumor dedifferentiation and progression to poorly differentiated and anaplastic carcinoma, which is likely to contribute to less favorable prognosis for these patients.

The prognostic implications of *BRAF* mutation could be of particular importance in papillary microcarcinomas, which are incidentally discovered tumors of ≤ 1 cm in size. These tumors are commonly found in thyroid samples removed for larger benign nodules. Most of the microcarcinomas are indolent tumors cured by surgical resection, although some of them demonstrate metastatic behavior, recur and may lead to patient death, and those require more aggressive treatment.[104] The role of *BRAF* mutation in defining a subset of aggressive papillary microcarcinomas remains to be fully defined. However, several recent studies have demonstrated that *BRAF* mutation in thyroid microcarcinomas correlates with either high rate of extrathyroidal tumor extension or lymph node metastasis or both of these features.[105–107]

RAS

The role of *RAS* mutation in predicting more aggressive tumor behavior is not well defined. As this mutation is also found in benign follicular adenomas, the *RAS* status by itself cannot be used for tumor prognostication. Some evidence exists, however, that *RAS* mutations in invasive follicular and papillary carcinoma may correlate with more unfavorable prognosis, although this correlation is far from being conclusively established.

Several studies have found a significant correlation between *RAS* mutation and metastatic behavior of follicular carcinomas, especially with respect to bone metastases, which may be due to the role of *RAS* mutation in promoting tumor dedifferentiation and transformation to anaplastic carcinoma.[46,48,108,109] The association between mutant *RAS* and more aggressive behavior of papillary carcinoma has been reported in a series of 91 tumors followed up on average for 14 years, in which *RAS* mutation was found to correlate with distant metastasis and significantly higher mortality rate.[47] On the other hand, *RAS* mutations are frequently found in encapsulated follicular variant of papillary carcinoma, the tumor with an indolent behavior.[51,110] Therefore, it is likely that *RAS* mutations mark a subset of widely invasive well-differentiated thyroid carcinomas, which are prone to metastatic spread and dedifferentiation and therefore entail higher mortality, but this mutation can not be used as a universal prognostic marker for all types of thyroid cancer.

Disclosure/conflict of interest

The author declares no conflict of interest.

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