TITLE: Molecular Pathology and Thyroid FNA

Running Title: Molecular pathology and its relevance to thyroid FNA

David Nigel Poller and Sharon Glaysher

Department of Pathology & Cancer Laboratory, Queen Alexandra Hospital, Cosham, Portsmouth, PO6 3LY, United Kingdom

Correspondence to Dr D Poller MD FRCPth, Consultant Pathologist, Department of Pathology, Queen Alexandra Hospital, Cosham, Portsmouth, PO6 3LY, UK.

Phone (44)(0) 2392 286458, Fax (44)(0) 2392 286493, Email david.poller@porthosp.nhs.uk

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Both authors declare that they have no conflict of interest relating to this work.

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Abstract: This review summarises molecular pathological techniques applicable to thyroid FNA. The molecular pathology of thyroid tumours is now fairly well understood. Molecular methods may be used as a rule-in test for diagnosis of malignancy in thyroid nodules e.g. *BRAF* V600E point mutation, use of a 7 gene mutational panel (*BRAF* V600E, *RAS* genes, *RET/PTC* or *PAX8/PPARG* rearrangement), or as a comprehensive multigene next-generation sequencing panel e.g. ThyroSeq v2. Molecular methods can also be applied as rule-out tests for malignancy in thyroid nodules e.g. Afirma or ThyroSeq v2 or as markers of prognosis e.g. TERT promoter mutation or other gene mutations including *BRAF* V600E, *TP53* & *AKT1* and as tests for newly defined tumour entities such as NIFTP, or as a molecular marker(s) for targeted therapies. This review describes practical examples of molecular techniques as applied to thyroid FNA in routine clinical practice.
Introduction

There have been major advances in molecular pathological techniques in the last few years, particularly with the introduction of next-generation sequencing for DNA and RNA. [1] Knowledge of the pathology of thyroid tumours has also evolved. Molecular diagnosis for thyroid nodules can be used for differential diagnosis of a thyroid nodule, patient prognostication, and as a molecular marker for targeted therapy. This review will concentrate on the use of molecular methods for diagnosis and prognostication. The molecular pathology of thyroid tumours is now comparatively well understood. [2, 3] Thyroid tumours are thought to arise as a result of multiple mutational events. Although thyroid nodules are extremely common most thyroid nodules are benign. The risk of thyroid malignancy in any given thyroid nodule also depends on the patient’s age, genetics, and previous history including radiation exposure. Metastatic tumours to the thyroid gland are also important as these are identified in 1.4-3.0% of suspected thyroid cancer patients. [4]

Papillary Thyroid Carcinoma

Papillary thyroid carcinoma (PTC) accounts for 85% of thyroid carcinomas; classical type or variants of classical type; tall cell, columnar cell, diffuse sclerosing, Warthin-like, or follicular variant of PTC. Classical type PTC shows somatic mutations in the mitogen activated protein kinase (MAPK) pathways due to RAS or BRAF mutations, or re-arrangements of RET or NTRK1. Papillary carcinomas usually show point mutations, BRAF mutations in 40-45%, RET/PTC rearrangements in 10-20% or RAS point mutations in 10-20%. [3] Small PTC tumours localised to the thyroid gland have excellent prognosis. Larger PTC tumours or tumours showing extrathyroidal extension or lymphatic or vascular invasion have adverse prognosis. Multiple gene mutations in the same tumour, e.g. BRAF V600E and TERT promoter, or PIK3CA, AKT1, TP53 mutations in addition to BRAF V600E mutation alone predict adverse prognosis. [5-8]. Of interest to cytology is that the aggressive variants of PTC, as well as showing cytological and histological differences to classical PTC, also show differing molecular profiles.
Tall cell variant of PTC shows higher frequency (up to 90% for \textit{BRAF V600E}) and \textit{TERT} promoter (31%) mutation compared to much lower rates seen in conventional PTC. [9, 10] Columnar cell variant of PTC is rare. \textit{BRAF V600E} mutations are found in one third of these tumours. [11] In hobnail variant PTC \textit{BRAF V600E} mutations are identified in a high percentage of cases, [12] [13] one study showed a 94.1% rate of \textit{BRAF V600E} mutation. [14] In diffuse sclerosing variant which is uncommon and typically affects younger patients \textit{RET/PTC} rearrangements are found in a high percentage of cases with \textit{BRAF V600E} mutations rare in the range of 0-61% although most studies suggest zero or very low rates of \textit{BRAF V600E} mutation. [15] \textit{ALK} translocations are said to be more common in diffuse sclerosing variant PTC, seen in one series in 3 of 14 \textit{ALK} translocated PTC cancer cases although \textit{ALK} fusions did not predict more aggressive tumour behaviour.[16] Solid variant of PTC is a rare variant defined by a solid/trabecular growth pattern. Foci of solid variant PTC were identified in papillary carcinomas in a small cohort of Chernobyl patients exposed to ionising radiation and were associated with \textit{RET/PTC3} or \textit{ETV6/NTRK3} rearrangements[17]. As the newly described mammary analogue secretory carcinoma (MASC) of the thyroid characteristically shows \textit{ETV6/NTRK3} fusion it has been suggested that some \textit{ETV6/NTRK3} fusion solid type PTC might be related to MASC tumours.[18] A series of oncocyic variant of PTC showed \textit{BRAF V600E} mutations in 6 of 15 cases (40%).[19] Macrofollicular variant of PTC is extremely rare with less than 80 cases reported. The very few cases studied have shown no evidence of \textit{BRAF} or \textit{RAS} mutations.[20] Cribriform-morular variant of PTC is an interesting variant, occurring predominantly in young females, either sporadically in patients with germline \textit{APC} mutations or in ~40% of cases it is associated with familial adenomatous polyposis. [21] While the results reported in the literature vary mutations of \textit{CTNNB1}, the gene encoding β catenin, or \textit{APC} mutations are most commonly found, or \textit{PIK3CA} mutations. \textit{KRAS} mutations, \textit{TERT} promoter mutation, and \textit{RET/PTC} mutations are rare and \textit{BRAF} mutations have not been found. \textit{Wnt} pathway activation is caused by germline \textit{APC} mutations, or somatic \textit{APC} mutations, and/or somatic \textit{CTNNB1} mutations. Aberrant positive nuclear β catenin staining can be identified in tumour cells.
**Follicular Variant of Papillary Thyroid Carcinoma (FVPTC)**

FVPTC typically shows a molecular type similar to that of follicular carcinoma and follicular adenoma with *RAS* mutations, *BRAF* K601E mutations, and *PAX8/PPARG* rearrangements which are common in follicular carcinomas and rare in classical papillary thyroid carcinoma. [22]

**Follicular Thyroid carcinoma (FTC)**

FTC account for around 10% or less of thyroid carcinomas. *BRAF* V600E mutations are very unusual although *BRAF* K601E mutations may occur. FTC are similar to that of follicular adenoma with mutations of *RAS* genes in approximately 40-50%, *PAX8/PPARG* rearrangement in 30-35% of cases, *PTEN* point mutations or small deletions in 5-10% and *PIK3CA* point mutations in 5-10%. [23] The oncocytic (also known as Hurthle cell) subtype of follicular carcinoma shows a much lower frequency of *RAS* point mutations (10-15%) and *PAX8/PPARG* rearrangements (0-5%) and absence of *PIK3CA* and PTEN point mutations. [24]

**Poorly Differentiated Carcinoma (PDTC) and Anaplastic Carcinoma (ATC)**

PDTC is rare, accounting for less than 1% of thyroid cancers. *RAS* mutations seen in around 30%, *BRAF* in 15%, *TP53* and β–catenin in around 25 to 30%. Anaplastic carcinoma typically shows high frequency of *RAS* (20-40%), *BRAF* V600E (20-40%), *TP53* (50-80%) and beta-catenin (5-60%) mutations. Anaplastic carcinoma is also characterised by other mutations e.g. *PIK3CA, PTEN, AKT1* and *APC* mutations, and fusions of *ALK* and other genes. [25]

**Medullary Thyroid Carcinoma (MTC)**

MTC comprises 2-4% of thyroid malignancies. Most MTC cases are sporadic although familial cases inherited in an autosomal dominant pattern comprise 15 to 30% of cases; MEN 2A, MEN 2B, and familial medullary thyroid carcinoma. Medullary carcinoma shows by *RET/PTC* translocations in more than 95% of cases. Familial MTC are associated with gain-of-function mutations in the *RET* gene.
Somatic RET mutations occur in 30-60% of sporadic medullary carcinomas and RAS mutations occur in sporadic medullary carcinomas. RAS mutations appear non-overlapping with RET and RAS gene mutations. [3]

**NIFTP Tumours**

Circumscribed or encapsulated follicular variant of papillary thyroid carcinoma without capsular or vascular invasion which until recently comprised approximately 20% of all newly diagnosed thyroid cancers in North America and in some centres elsewhere has now been redesignated as NIFTP, ‘non-invasive follicular thyroid neoplasm with papillary like nuclei’. These lesions have very low risk of recurrence and are longer designated as carcinomas. [26] NIFTP tumours are RAS driven tumours similar to follicular carcinoma and rarely show BRAF V600E mutations (if the very strict diagnostic criteria are applied) and frequent presence of RAS mutations, PAX8/PPARG translocations or THADA fusions. While some studies show relatively frequent BRAF V600E mutation rates particularly studies from Korea, in lesions said to show features of NIFTP, [27] in the authors’ view it is likely that these high reported rates of BRAF V600E mutation were achieved because of failure to apply strict pathological diagnostic inclusion and exclusion criteria for diagnosis of NIFTP tumours. NIFTP tumours are classified as ‘suspicious’ using Afirma. [28, 29] The use of the revised NIFTP terminology is having a major impact on diagnostic thresholds for cytological diagnosis of all categories of thyroid FNA.

**Pre-operative Molecular Diagnosis for Thyroid FNA**

Molecular testing can either be used as a ‘rule-in’ or a ‘rule-out’ test for malignancy. [30] The performance of the test depends on the positive predictive value (PPV) and the negative predictive value (NPV) for malignancy although the PPV and NPV also depend on the pre-test probability of malignancy in any given sample. Individual institutional rates of malignancy vary depending on the relevant FNA category. Around 25% of thyroid aspirates are classified as indeterminate; Bethesda
category III and IV, in the United Kingdom broadly equivalent to Thy3a and Thy3F and in Italy as TIR3a or TIR3f with a published risk of malignancy ranging from 5-30%. [31]

**Approaches to Molecular Diagnosis of Thyroid FNA**

**Afirma**

The *Afirma Gene Expression Classifier (GEC)* (Veracyte, South San Francisco, USA) is a proprietary test that uses micro-RNA mRNA profiles for diagnosis of indeterminate nodules. [32] This test is widely used in the USA but is not currently routinely commercially available in Europe or elsewhere. Two dedicated needle passes are collected into vial of RNA preservative with also FNA passes for routine cytology. This test is most helpful for indeterminate FNA when surgery is not desirable, e.g. small nodule size, patient preference, or absence of symptomatic goitre. An initial 25 gene screen for expression profiles of less common entities in the thyroid such as metastatic lesions, parathyroid tissue, medullary thyroid carcinoma and oncocytic lesions is followed by the main 142 gene expression classifier. If one of the six preliminary screening cassettes is triggered the sample is reported as a ‘suspicous’. A blinded prospective multicentre trial of 265 nodules with indeterminate cytology and histological follow-up was reported in 2012. [32] Centres using *Afirma* use it as a ‘rule-out’ test for indeterminate thyroid nodules, that is Bethesda class III and IV nodules. A benign result implies a risk of malignancy of around 5-6%, with meta-analysis reporting a pooled sensitivity of 95.7% and a pooled specificity of 30.5%. [33] A suspicious for malignancy result has the negative predictive value of around 85%. The 2012 study showed a low positive predictive value for malignancy for indeterminate nodules, 38% for Bethesda category III and 37% for Bethesda category IV. The high NPV of a benign GEC result in category IV nodules reduces the risk of malignancy to a range of 5 to 6% which is comparable to that of a benign aspirate so for category IV FNA a benign GEC test result removes the need for surgery. Studies of the GEC show very few carcinomas in the few cases that have undergone excision after a benign GEC diagnosis; Nishino in 2016 reported that 8.5% of benign GEC cases had undergone surgery but only three benign GEC cases were malignant; a
6 mm papillary carcinoma, a 32 mm follicular carcinoma and a 28 mm cystic papillary carcinoma. [30] Oncocytic FNA’s show a lower risk of malignancy with suspicious GEC results. In 2014 Veracyte added the Afirma MTC and Afirma BRAF, both mRNA classifiers, for identification of the gene expression signature for medullary carcinoma [34] and BRAF.[35]

**BRAF V600E Mutation Alone**

*BRAF* V600E mutation is >99% specific for diagnosis of thyroid cancer. *BRAF* V600E testing is most useful for higher risk FNA’s when nuclear features of papillary carcinoma are suspected but the cytological diagnosis remains uncertain, i.e. a diagnosis of Bethesda category IV or V (Thy3F or Thy4) where a confident diagnosis of malignancy cannot be made. In these cases companion testing for *BRAF* V600E using a PCR technique will identify *BRAF* V600E mutations typically in 40-50% of Bethesda Category V/Thy4 FNA’s. A meta-analysis of 47 studies of *BRAF* V600E testing of FNA cytology specimens showing a pooled sensitivity for *BRAF* V600E testing in thyroid FNA of 52% (95% CI 39%-64%). Sensitivity when reported in individual studies was 100% but pooled sensitivity for indeterminate FNA (category III to V) in the 6 studies where this could be calculated was 31% (95% CI, 6%-56%). [36] *BRAF* V600E testing is also useful because NIFTP tumours, which are follicular derived *RAS*-driven thyroid lesions rarely, if strict diagnostic criteria are applied, show *BRAF* V600E mutations and so the presence of a *BRAF* V600E mutation can be used to exclude a NIFTP tumour in FNA aspirates when nuclear features suggestive of papillary carcinoma are present.

**7 Gene Test**

The so-called ‘7 Gene Test’, a panel combination of *BRAF* V600E, *PAX8/PPARG*, *RET/PTC*, *HRAS*, *KRAS* & *NRAS* [37] has been shown to have greater sensitivity and specificity than *BRAF* V600E testing alone. This approach is one of the methods suggested in *The American Thyroid Association Statement on Application of Molecular Profiling for Thyroid Nodules: Current Impact on Perioperative Decision Making.*[38] While this offers more information than *BRAF* V600E testing alone in practice
the results are often less clear e.g. a study from Europe published in 2017 showed that the application of the seven gene panel in a routine primary referral setting did not improve the presurgical diagnosis of thyroid FNA. [39] The reasons for this were the presence of RAS gene mutations in adenomas as well as malignant tumours and the low frequency of RAS mutation positive carcinomas in this study.

**Full Next Generation Sequencing (NGS) Panel : Thyroseq2**

*ThyroSeq v2* is both a ‘rule-in’ and ‘rule-out’ test. The *ThyroSeq v2* panel uses NGS for simultaneous analysis of multiple genes. *ThyroSeq v2* tests for point mutations and small insertions/deletions in 14 genes, 42 types of gene fusions, and expression levels of 16 genes. *Thyroseq 2* detects RET mutations (medullary thyroid carcinoma) and also *TERT* promoter mutation, a mutation, which is both a diagnostic and prognostic marker, and present at increased frequency in aggressive tumours such as poorly differentiated carcinoma and anaplastic thyroid carcinoma. *TERT* promoter mutation is associated with increased risk of distant metastases, persistent disease, and cancer-specific mortality. *TP53*, *PIK3CA*, and *AKT1* gene mutations are associated with aggressive behaviour and tumour progression, particularly when found in combination with early driver events like *BRAF* or *RAS* mutations. *Thyroseq2* detects an extended list of gene fusions including beyond the most common *RET/PTC1*, *RET/PTC3*, and *PAX8-PPARG* fusions including additional fusions of *RET*, *BRAF*, *NTRK1* and 3, *ALK*, and other genes. Gene expression markers also assess the quality of samples, and specifically the proportion of thyroid follicular cells, and also the expression of the calcitonin and parathyroid hormone genes, which assists in diagnosis of medullary thyroid carcinoma and parathyroid lesions. *ThyroSeq v2* was initially validated in a single institutional combined retrospective and prospective study of 143 Bethesda Category IV thyroid nodules [40] with a specificity of 93%, sensitivity of 90%, PPV of 83% and NPV of 96%. [40] In a follow-up, single institution, prospective study of 465 Bethesda Category III thyroid nodules a sensitivity of 90.9%, specificity of 92.1%, PPV of 76.9%, and NPV of 97.2% was seen. [41]. Using a comprehensive NGS
panel such as ThyroSeq 2 identification of RAS or a RAS-like mutation implies a high probability (~80%) of either a low-risk cancer or NIFTP for which surgery can often be limited to thyroid lobectomy. Test positivity for an isolated BRAF V600E or other BRAF V600E-like mutation implies a very high (>99%) probability of cancer, expected to be of intermediate risk for disease recurrence. These patients may undergo total thyroidectomy or lobectomy, depending on the clinical situation. ThyroSeq 2 positivity for multiple mutations or high-risk mutations such as TERT is virtually diagnostic of cancer and increased risk of disease recurrence and possibly cancer-related mortality so these patients can benefit from total thyroidectomy.

**Practical applications**

A recently published Template for Reporting Results of Biomarker Testing for Patients with Suspected Thyroid Carcinoma produced by the College of American Pathologists gives a series of practical explanatory notes on the clinical significance of identified mutations of BRAF V600E, RAS, PIK3A, AKT1, TP53, CTNNB1, RET, ALK, NTRK1, NTRK3 and PPARG. [42] 5 to 10% of thyroid cancers do not appear to have a specifically identifiable driver gene mutation that can be identified using existing molecular techniques and there is an overlap in the mutational profiles of benign and malignant thyroid lesions particularly a problem with follicular adenoma and follicular carcinoma as both benign and malignant thyroid lesions may show identical point RAS gene mutations. A task force of the European Thyroid Association (ETA) has also just recently produced guidelines for thyroid nodule molecular fine needle aspiration diagnostics. [43]. The ETA guidelines conclude that molecular tests may help clinicians to drive patient care and surgical decision making if analysis is performed in specialised laboratories, requiring standardisation of performance characteristics, appropriate calibration, and analytical validation for clinical interpretation.

**BRAF V600E Mutation**
(i) Diagnosis of papillary carcinoma in higher risk FNA: In patients with Bethesda Category V/Thy4 FNA’s, molecular testing of the sample for BRAF V600E mutation is useful as approximately 50% of the specimens will show evidence of a BRAF V600E mutation and therefore management can be escalated to that of a category VI/Thy5 lesion malignant lesion.

(ii) As a test of exclusion for NIFTP tumours: As NIFTP tumours rarely show evidence of BRAF V600E mutations the presence of a BRAF V600E mutation virtually excludes a diagnosis of NIFTP.

(iii) Confirmation of the diagnosis of papillary thyroid carcinoma. This is useful in paucicellular FNA’s or cell block samples or supernatant samples from the thyroid as BRAF V600E mutation is almost 100% specific for a diagnosis of papillary thyroid carcinoma. This is most helpful if the patient is known to have pre-existing BRAF V600E mutation positive thyroid carcinoma and the sample is a suspected recurrent or metastatic thyroid carcinoma.

(iv) Morphological Phenotyping. BRAF V600E mutated thyroid carcinoma cells have a fairly characteristic morphology in many cases with pink cytoplasm evident in both cytological and histological preparations. This effect was noted initially in histological specimens by the presence of moderate to abundant eosinophilic cytoplasm in tumour cells that were polygonal or somewhat tall but did not quite meet the criteria for tall cell variant so called ‘plump pink cells’. [44] Plump pink cells are extremely common in BRAF V600E-mutated tumours and this has now been confirmed in cytological preparations with cytologically plump cells with sickle shaped nuclei in the majority of cases. [45, 46]

7 Gene Test

(v) The reported results of these tests in the literature are conflicting as the results range from 18-100% sensitivity, 86-100% specificity, 56-100% negative predictive value and 19-100% positive predictive value depending to the clinical setting, that is the risk of malignancy of the individual FNA being submitted for molecular testing and the institution concerned. The ETA guidance concludes...
that for cytologically indeterminate nodules, *BRAF*, *RET/PTC*, *PAX8/PPARG* and *RAS* mutational analysis should be considered although the significance of *RAS* mutations needs to be clarified. [43]

**Afirma**

(vi) As a ‘rule-out’ test of exclusion for malignancy Afirma has been deployed in North America with high success as a test of exclusion for indeterminate; Bethesda category III and IV nodules.

**Comprehensive NGS Panel.**

(vii) *Thyroseq v2 can be used* as a highly effective ‘rule-in’ and ‘rule-out’ test for malignancy.

**Tumour prognostication**

(viii) Thyroid FNA can be used as a prognostic indicator if specific gene mutations or phenotypic alterations can be identified. While *BRAF* V600E mutation alone may confer slightly adverse prognosis in any given case, the adverse effect of this mutation alone is small although recent evidence indicates that a combination(s) of *BRAF* V600E mutation with other gene mutations e.g. *BRAF* V600E and *TERT* promoter mutation, or coexistent mutations of *BRAF* V600E and *TP53, PIK3CA, AKT1* or *ALK* fusions convey significant adverse prognosis all of which can be identified using multigene next-generation sequencing panels.

**Conclusion**

Molecular methods have become important as diagnostic and developmental tools in thyroid disease and in the understanding of the pathogenesis of thyroid cancer, in diagnosis, prognosis, and treatment. Many of these methods can be used preoperatively on small samples of DNA or RNA or micro-RNAs from thyroid FNA aspirates if resources are available although given the complexities of testing and test validation most of the methods described are most suited to a specialised referral laboratory setting.
Figure Legends

Table 1

Mutational profiles seen in thyroid tumours. Note that the profile of follicular adenoma and NIFTP overlaps considerably with encapsulated follicular variant of papillary carcinoma and follicular carcinoma (follicular-like tumours) whereas classical type papillary carcinoma shows a mutational profile with \textit{BRAF} V600E mutation.

Figure 1

Illustration re-drawn from the 2015 \textit{American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Carcinoma; Risk of Structural Disease Recurrence in Patients without Structural Identifiable Disease after Initial Therapy} showing how molecular profiling; \textit{BRAF} V600E mutation & \textit{TERT} promoter mutations can be used to prognostically profile thyroid nodules.

Figure 2

Putative molecular pathogenic pathway for thyroid tumours. Classical type papillary carcinoma is typically \textit{BRAF} V600E driven, whereas follicular adenoma, follicular thyroid carcinoma and NIFTP are \textit{RAS} driven lesions.
References


11. Chen JH, Faquin WC, Lloyd RV, Nose V. Clinicopathological and molecular characterization of nine cases of columnar cell variant of papillary thyroid carcinoma. Mod Pathol. 2011;24(5):739-49.


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Differentiated Follicular Derived Thyroid Cancer
(from American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer 2015)

Risk of Structural Disease Recurrence
(in patients without structurally identifiable disease after initial therapy)

**HIGH RISK**
- Gross extrathyroidal extension, incomplete
- Tumor resection, distant metastases or lymph node >3cm

**INTERMEDIATE RISK**
- Aggressive histology, minor extrathyroidal extension, vascular invasion, or >5 involved lymph nodes (0.2-3cm)

**LOW RISK**
- Intrathyroidal DTC
- <= 5 LN micrometastases (<0.2cm)

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FTC, extensive vascular invasion (~30-55%)
pT4a gross ETEx (~30-40%)
pN1 with extranodal extension, >3 LN involved (~40%)
PTC, >1cm, TERT mutated, +/- BRAF mutated (~40%)
pN1, any LN >3cm (~30%)
PTC, extrathyroidal, BRAF mutated (~10-40%)
PTC, vascular invasion (~15-30%)
Clinical N1 (~20%)
pN1, >5 LN involved (~20%)
Intrathyroidal PTC, <4cm, BRAF mutated (~10%)
pT3 minor ETEx (~3-8%)
pN1, all LN <0.2cm (~5%)
pN1, <=5 LN involved (~5%)
Intrathyroidal PTC, 2-4cm (~5%)
Multifocal PMC (~4-6%)
pN1 without extranodal extension, <=3 LN involved (2%)
Minimally invasive FTC (~2-3%)
Intrathyroidal, <4cm BRAF wild type (~1-2%)
Intrathyroidal unifocal PMC, BRAF mutated (~1-2%)
Intrathyroidal, encapsulated, FVPTC (~1-2%)
Unifocal PMC (~1-2%)
# Putative Molecular Pathogenesis of Thyroid Tumours

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Diagram showing the molecular pathogenesis of thyroid tumours with different growth patterns and nuclear features, along with associated oncogenes and tumour types.