Classification of follicular cell-derived thyroid cancer by global RNA profiling

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Abstract

The incidence of thyroid cancer is increasing worldwide and thyroid nodules are a frequent clinical finding. Diagnosing follicular cell-derived cancers is, however, challenging both histopathologically and especially cytopathologically. The advent of high-throughput molecular technologies has prompted many researchers to explore the transcriptome and, in recent years, also the miRNome in order to generate new molecular classifiers capable of classifying thyroid tumours more accurately than by conventional cytopathological and histopathological methods. This has led to a number of molecular classifiers that may differentiate malignant from benign thyroid nodules. Molecular classification models based on global RNA profiles from fine-needle aspirations are currently being evaluated; results are preliminary and lack validation in prospective clinical trials. There is no doubt that molecular classification will not only contribute to our biological insight but also improve clinical and pathological examinations, thus advancing thyroid tumour diagnosis and ultimately preventing superfluous surgery. This review evaluates the status of classification and biological insights gained from molecular profiling of follicular cell-derived thyroid cancers.

Key Words

- thyroid
- cancer
- classification
- global RNA profiling

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Introduction

Thyroid cancer is the most common cancer of the endocrine organs and its incidence has increased over recent decades (Kondo *et al.* 2006). The majority of thyroid carcinomas derive from follicular thyroid cells, also known as thyroid epithelium or thyroid principal cells, responsible for thyroid hormone production and secretion (Fig. 1). The follicular thyroid cell develops from an outgrowth of the pharyngeal endoderm in the region of the tongue (foramen caecum). Approximately 3% of carcinomas are medullary carcinomas, which originate from parafollicular or C-cells that arise from the fourth branchial pouch and produce and secrete calcitonin.

The most common follicular cell-derived carcinomas are papillary thyroid carcinomas (PTCs), which account for 80–90% of all thyroid cancers and have a 5-year survival above 90% (Kondo *et al.* 2006). The second most common follicular cell-derived carcinomas are follicular thyroid carcinomas (FTCs), accounting for ~10% of thyroid cancers (Kondo *et al.* 2006). The frequency of distant metastases at the time of diagnosis of FTC is up to 20%. Nevertheless, the prognosis of FTC is still favourable with a 5-year survival close to 90% (Kondo *et al.* 2006). FTCs are the malignant counterparts of benign follicular thyroid adenomas, which represent the most common follicular

Figure 1

Schematic model of the stepwise dedifferentiation of follicular cell-derived thyroid cancer. Somatic mutations were depicted based on reported frequencies (Kondo *et al.* 2006, Nikiforov & Nikiforova 2011).

cell-derived tumours of the thyroid (Faquin 2008). The sole morphological feature differentiating follicular thyroid adenomas from FTCs is the lack of invasion (capsular and/or vessel), invasion being the diagnostic criterion for FTCs (Faquin 2008). The diagnostic reproducibility of FTC is a challenge, and even among expert thyroid pathologists, the inter-observer and intra-observer reproducibility is low. One study found the agreement between five thyroid pathologists' initial diagnosis and the final consensus diagnosis of FTCs to be between 0.11 and 0.69 (Franc *et al.* 2003). Poorly differentiated and undifferentiated thyroid cancer, also known as anaplastic thyroid carcinoma (ATC), represents a small subset of follicular cell-derived cancers with a poor prognosis. The growth patterns of ATC and of poorly differentiated thyroid cancer are highly invasive and more than 50% of patients have distant metastases at the time of diagnosis (Kondo *et al.* 2006). The 5-year survival rate of ATC is no more than 5%, illustrating why ATC is considered one of the most fatal human cancers (Kebebew *et al.* 2005).

Several somatic point mutations and chromosomal rearrangements have been identified in follicular cell-derived thyroid cancer (see Fig. 1 for an overview). Mutations are present in genes belonging to the MAPK signalling pathway such as *BRAF*, *HRAS*, *KRAS* and *NRAS* as well as *RET/PTC* and *TRK (NTRK1)* fusion proteins (Nikiforov 2011).

Thyroid fine-needle aspirate (FNA) is the key test in the preoperative evaluation of euthyroid patients with thyroid nodules (Gharib *et al.* 2010). The possible

cytopathological diagnoses determined by FNA are benign, malignant, non-diagnostic and suspicious/indeterminate cytology (follicular neoplasia; Yassa *et al.* 2007). The latter category of lesions represents one of the greatest challenges to the attending physician as well as researchers in the field of thyroid cancer.

Recent studies have evaluated the diagnostic contribution of preoperative mutational testing of FNA with indeterminate cytology (Moses *et al.* 2010, Ohori *et al.* 2010, Filicori *et al.* 2011, Nikiforov *et al.* 2011). A common finding was that testing for well-known somatic mutations improved the diagnostic value. As somatic mutations were, however, only present in fewer than half of the malignant cases, the overall ability to rule out malignancy was not satisfactory.

In recent years, several studies have employed gene expression profiling to characterise thyroid cancer. The focus has mainly been on the diagnostic dilemma of follicular cell-derived neoplasia and on various classification models, as well as on thyroid cancer carcinogenesis (Eszlinger *et al.* 2008). Moreover, studies of epigenetic regulation, centred on expression profiling of microRNAs (miRNAs of 21–25 nucleotides), have appeared (de la Chapelle & Jazdzewski 2011). It is noteworthy that a recent cost-benefit analysis favoured the addition of molecular testing of follicular cell-derived thyroid neoplasia at the preoperative stage, as this would lead to a more effective clinical management of these patients (Li *et al.* 2011).

Studies of expression levels of mRNAs by microarray analysis have been the primary way to investigate the transcriptome. It was recently recognised that only a fifth of the transcriptome consists of protein-coding mRNA (Kapranov *et al.* 2007). Thus, a great part of the mammalian transcriptome comprises noncoding RNAs, that in addition to the well-known noncoding RNAs of ribosomal RNAs and miRNAs, also comprise long-noncoding RNAs (>200 nucleotides). Future studies are likely to clarify the transforming potential of long noncoding RNAs as well as their value in thyroid cancer classification. This review focuses on the advantages of the addition of high-throughput technologies for transcriptional analyses with regard to tumour classification and the elucidation of thyroid carcinogenesis.

Tumour biology of thyroid cancer as examined by microarray mRNA profiling

Global mRNA microarray analysis is an efficient way of attaining a general idea of the genetic profiles of specific cancers. The following review of thyroid cancer profiling

reveals that a unique genetic profile of each of the many pathohistological variants of follicular cell-derived thyroid cancer has not been established. This could be due to an inherent morphological heterogeneity as well as different time points for surgery of these relatively slow-growing cancers. Figure 2 illustrates the variance across 92 different thyroid samples based on the entire transcriptome depicted in a principal component analysis plot. In short, FTCs, follicular adenomas (FAs), fetal adenomas (FEAs) and PTCs do not form separate clusters but appear to be mingled together in a large group with little variance. Only the two extremes, represented by the ATCs and the hyperplastic nodules (HYPs)/nodular goitres, are clustered in well-defined groups. This plot illustrates the difficulties in obtaining a clear mRNA profile from each follicular cell-derived thyroid tumour variant. Some of these difficulties might have a technical origin: differences in RNA extraction methods, tissue types and platform design may affect the final genetic profile. This is also evidenced by the poorer performances of molecular classifiers based

on frozen tissue and subsequent validation with FNAs where the pre-processing procedures are different.

To gain insight into the carcinogenic processes underlying follicular cell-derived thyroid cancer, several studies have taken advantage of global RNA analyses. In particular, the use of microarray has been widespread; this, in short, consists of hybridisation of labelled RNA to an array of immobilised, complementary probes. The high-throughput array technique detects differences in expression levels of most of the transcriptome but lacks sufficient sensitivity for the detection of low abundance transcripts (Gupta *et al.* 2011). Several important transcripts encoding transcriptional regulators are expressed at low levels. Recent methodology, known as 'RNA-Seq', involves the conversion of RNA into cDNA with adaptors for deep-sequencing technology. RNA-Seq is highly sensitive and quantitative and is unbiased as it can quantify both known and unknown transcripts (Ozsolak & Milos 2011). This technique is likely to add to the present understanding of thyroid carcinogenesis.

The microarray study by Hawthorn *et al.* (2004) found the cellular adhesion genes *TIMP1* (tissue inhibitor of metalloproteinase 1) and *SERPINA1* to be up-regulated in PTC. *SERPINA1* has recently been proposed as a consistent mRNA marker for PTC in a microarray meta-analysis of more than 40 PTC samples and 82 independent validation samples (Vierlinger *et al.* 2011). Moreover, this study showed the extracellular matrix pathway to be strongly activated, indicating an important role of tumour–stroma interaction in the carcinogenesis of PTC (Vierlinger *et al.* 2011). The performance of the diagnostic accuracy for PTC is specified in Table 1. Based on several online microarray results with relatively small sample sizes, another meta-analysis by Vizioli *et al.* (2010) recently confirmed down-regulation of *IGFBP7* in follicular cell-derived cancers (PTC and FTC). In addition, functional studies showed *IGFBP7* to be an oncosuppressor with effects related to apoptosis. Thus, by taking advantage of larger microarray datasets accessible online, it is possible to strengthen the findings with functional studies.

Because the morphological features of PTC, FTC and follicular variant of PTC (FVPTC) are similar, mRNA profiling studies have been carried out to improve the understanding of the different molecular features of these cancers. Aldred *et al.* (2004) compared PTC and FTC profiles and found overexpression of *CITED1*, *CLDN10* (encoding claudin 10 that plays a role in cell adhesion activity) and *IGFBP6* (encoding insulin-like growth factor-binding protein 6 that alters the interaction of IGFs with their cell surface receptors) in PTC. Interestingly, *CLDN10*

Figure 2

A principal component analysis based on the entire transcriptome from 92 different thyroid samples. The three first principal components are designated with the numbers 1–3. The percentage of the total variance described by each of the three principal components is given in parentheses at each axis. FA, light blue; follicular carcinoma (FC), orange; FEA, green; anaplastic carcinoma (AC), red; papillary carcinoma (PC), dark blue; nodular goitre (NG), pink; normal thyroid (NT), white. Adapted from Borup R, Rossing M, Henao R, Yamamoto Y, Kroghdal A, Godballe C, Winther O, Kiss K, Christensen L, Hogdall E, *et al.* 2010 Molecular signatures of thyroid follicular neoplasia. *Endocrine-Related Cancer* **17** 691–708, 2010 with permission from Society for Endocrinology & BioScientifica Ltd.

Table 1 Studies of mRNA-based classification

Type of tumours	Sample type	Method	Transcripts	Diagnostic classes	Diagnostic performance ^a	References
14 PTC/FVPTC, 14 FA, 7 HYP + 7 independent samples for validation	42 fresh frozen thyroid tissues	Microarray	1149 differentially expressed transcripts	Malignant vs benign	Sens. = 93%	Finley et al. (2004a)
10 HYP, 16 FA, 13 FVPTC, 11 PTC, 9 FTC and other variants	62 fresh frozen thyroid tissues	Microarray	627 differentially expressed transcripts	Malignant vs benign	Spec. = 100% Sens. = 92%	Finley et al. (2004b)
24 FTC, 17 FA, 12 HYP, 2 NTT	55 fresh frozen thyroid tissues	Microarray and QRT-PCR (validation samples, n = 31)	PCSK2, PLAB, CCND2	FTC vs benign	Spec. = 96% Sens. = 100%	Weber et al. (2005)
13 FA, 10 HYP	23 fresh frozen thyroid tissues	Microarray	402 transcripts	FA vs HYP	Spec. = 95% Sens. = 84.6%	Finley et al. (2005)
23 PTC, 17 NTT and 10 other variants	50 fresh frozen tissues + 34 validation samples	Microarray	19 transcripts	PTC vs NTT/HYP/FA	Spec. = 100% Sens. = 85.7%	Jarzab et al. (2005)
13 HYP, 13 FA, 13 PTC, 13 FVPTC, 13 FTC and other variants	94 fresh frozen thyroid tissues + 31 validation samples	Microarray and QRT-PCR (validation samples, n = 31)	75 transcripts	Malignant vs benign	Spec. = 100% Sens. = 73%	Prasad et al. (2008)
18 FTC, 34 FA, 4 ATC, 2 PTC, 9 HYP, 2 NTT	69 fresh frozen thyroid tissues + online available data (n = 23)	Microarray	117 transcripts	FTC vs FA	Spec. = 82% PPV = 78% Sens. = 94%	Borup et al. (2010)
FTC, FA, PTC, HTA, HTP, HYP	137 FNAs (<i>in vivo/ex vivo</i>), 178 fresh frozen tissues (validation FNAs = 48)	Microarray	Not specified	Malignant/suspicious vs benign	Spec. = 96% PPV = 94% NPV = 96% Spec. = 84%	Chudova et al. (2010)
40 PTC, 34 NTT	74 online available data + 82 fresh frozen samples for validation	Microarray (GEO)	SERPINA1	PTC vs HYP	NPV = 96% Sens. = 98%	Vierlinger et al. (2011)
50 HYP, 10 FA, 23 PTC and other thyroid tumour variants	95 intraoperative FNA samples	QRT-PCR	HMGGA2, MRC2, SFN	Malignant vs benign	Spec. = 100% PPV = 100% NPV = 98% Sens. = 71%	Prasad et al. (2012)
265 indeterminate nodules	265 FNAs (<i>in vivo</i>)	Microarray	167 transcripts (142 + 25)	Malignant/suspicious vs benign	Spec. = 84% PPV = 65% NPV = 88% Sens. = 92% Spec. = 52% PPV = 47% NPV = 93%	Alexander et al. (2012)

GEO, gene expression omnibus; Sens., sensitivity; Spec., specificity.

^aDiagnostic accuracy for predicting malignancies.

was completely absent in FTC, and *IGFBP6*, caveolin-1 and caveolin-2 were down-regulated (Aldred *et al.* 2004). Caveolin-1 and -2 are co-expressed and encode proteins localised in caveolae as a part of the plasma membrane in most mammalian cells. Moreover, both genes may act as tumour suppressors. A subsequent study confirmed either a diverse molecular origin or a distinct genetic diversity from a common origin by identifying a number of differentially expressed transcripts in PTC vs FTC (cf. Fig. 2; Zhao *et al.* 2006).

One of the earliest microarray studies based on tissue samples from 19 FTCs and 14 adjacent normal thyroid tissues (NTTs) focused solely on the genetic signature of FTC and primarily identified loss of function; caveolin-1, caveolin-2, *GDF10* (regulates differentiation), and glypican-3 (may regulate growth) were ≥ 15 -fold repressed in FTC. Subsequent immunohistochemical analysis of a large set of follicular cell-derived cancers indicated loss of caveolin-1 to be characteristic for FTC, thus representing a potential biomarker (Aldred *et al.* 2003). Furthermore, Chevillard *et al.* (2004) showed a similar diversity of involved biological functions in FTC and FA. They identified changes in expression levels for transcripts of gene families involved in the cytoskeleton, cell adhesion, cellular matrix, immune response and stress-related and signal transduction. Eszlinger *et al.* (2005) pooled expression profiles from HYP and FA and identified 31 differentially expressed transcripts after comparison with the adjacent NTTs. Gene set enrichment analysis showed a significant enrichment of cell cycle-associated genes (cyclin B, cyclin D, cyclin H, *CDK1* and 7, *p21*, *RB1*, *PRKDC*, *MAD2L1*, *Cdc20* and *WEE1*, as well as transcription factors *DP1* (*TFDP1*), *E2F4* and *E2F5*) and G-protein kinase signalling. Moreover, Belge *et al.* (2008) showed an overexpression of *HMGA2* in FTC as well as in PTC. The *HMGA2* transcript encodes a DNA-binding protein known to be an architectural factor in the DNA unwinding process. They evaluated the potential of *HMGA2* as a stand-alone biomarker. Based on RNA extraction from archived formalin-fixed, paraffin-embedded samples ($n=64$) and subsequent QRT-PCR analysis, they determined a sensitivity and specificity for thyroid cancer of 96 and 94% respectively. In spite of these promising results, *HMGA2* is, however, not consistently up-regulated in FTCs, emphasising the need for a larger and more robust molecular classifier.

A later study conducted by our group not only classified but also characterised the expression profile of FTC. We showed FTC to be characterised by increased levels of mRNAs encoding proteins involved in DNA

replication and mitosis, *TOP2A*, aniline (*ANLN*), PDZ-binding kinase (*PBK*) and abnormal spindle-like microcephaly-associated protein (*ASPM*) (Borup *et al.* 2010). *TOP2A* is an enzyme that alters the topologic states of DNA in the transcription phase and its dysregulation can cause chromosomal instability. *TOP2A* is of particular interest as it represents a possible treatment target (Pritchard *et al.* 2008, O'Malley *et al.* 2009). Additionally, we found loss of transcripts encoding proteins involved in growth arrest and apoptosis such as FBJ murine osteosarcoma viral oncogene homologue B (*FOSB*), JUN proto-oncogene (*JUN*), *NR4A1* and *NR4A3* (nuclear receptor subfamily 4, group A, member 1 and member 3) (Borup *et al.* 2010). Members of the orphan nuclear receptor family, *NR4A1* and *NR4A3*, were studied both *in vivo* and *in vitro* by Mullican *et al.* (2007), who established their role as critical tumour suppressors in cancer. They showed that co-abrogation of *NR4A1* and *NR4A3* leads to acute myeloid leukaemia. A recent study by Lu *et al.* (2011) investigated the genes involved in the metastatic spread of thyroid carcinomas by generating a mouse that spontaneously develops metastatic FTC. By comparing the expression profiles of the tumour cells with hyperplastic thyroid cells from a wild-type mouse, the authors found more than a third of the 150 differentially expressed genes to be key regulators of metastasis, including olfactomedin 4 and epithelial membrane protein 2. The remaining genes encoded proteins involved in metabolism, intracellular trafficking, transcription regulation, post-transcriptional modification and cell-cell signalling.

Only a few studies have explored the rare but virulent ATC by microarray analysis. Onda *et al.* (2004) showed up-regulation of *GDI2* (localisation of Rab proteins), destrin (cell structure), *HSPA8* (proliferation and apoptosis) and stathmin (microtubules) in anaplastic cell lines and primary ATCs and down-regulation of the thyroid-specific transcript thyroglobulin, as well as phosphatidylethanolamine-binding protein-1 and proto-oncogene *c-FES* (*FES*), genes indicating reversal to a poorly differentiated phenotype. To gain knowledge regarding thyroid carcinogenesis, Montero-Conde *et al.* (2008) examined seven ATCs, six poorly differentiated thyroid carcinomas, seven FTCs and 24 PTCs by microarray. Apart from confirming previously reported aberrantly expressed transcripts, they found an overexpression of key regulators (*AKT2* (serine/threonine protein kinases), *SOS1* (proto-oncogenic receptor tyrosine kinase) and *FGFR1* (fibroblast growth factor receptor 1)) of the MAP kinase pathway as well as an under-expression of negative regulators (*PLA2G6* (calcium-independent phospholipase A2),

NF1 (neurofibromatosis-related protein NF-1) and *CASP2* (caspase 2)) in poorly differentiated thyroid carcinoma/ATC vs well-differentiated cancers (FTC/PTC). Moreover, the study revealed overexpression of mRNAs encoding proteins involved in the cell cycle, focal adhesion, regulation of the actin cytoskeleton and the TGF- β signalling pathway.

Tumour biology of thyroid cancer as examined by global miRNA profiling

miRNAs are noncoding and single-stranded RNAs of ~21–25 nucleotides. miRNAs regulate translation and stability of about one third of the mammalian protein-coding mRNAs by imperfect base pairing to the 3'-ends of their target mRNAs (Bartel 2004, 2009, Friedman *et al.* 2009). The expression of miRNAs is temporally and spatially controlled and miRNAs exhibit important functions during developmental stages as well as in mature cells (Schmittgen 2008). Moreover, miRNAs are aberrantly expressed in a variety of cancers (Rosenfeld *et al.* 2008). Many target mRNAs encode oncogenic proteins and tumour suppressors and are susceptible to dysregulated miRNAs that may play a causal role in malignant progression. The library of human miRNAs contains ~1000 unique mature miRNAs, which, in contrast to the transcriptome, is a more manageable number of genes to investigate. miRNAs are well preserved in frozen specimens, FNAs and formalin-fixed, paraffin-embedded samples, establishing them as attractive molecular objects of study in translational medicine. However, as miRNAs are considered as modulators of the transcriptome rather than major switches of gene expression, it is necessary to relate the aberrant miRNAs with their target mRNAs in order to analyse their significance in any cancer.

In a recent review, de la Chapelle & Jazdzewski (2011) proposed that the failure to find mutations in traditional protein-coding genes is not coincidental and hypothesised that changes in the expression of multiple noncoding RNA genes, e.g. miRNAs, might be an important mechanism in thyroid carcinogenesis. A very recent report by Yu *et al.* (2012) examined specific miRNAs in sera and thyroid tissues of patients with PTC or benign nodules. This was achieved by genome-wide sequencing of miRNAs, followed by QRT-PCR quantification. Serum let-7e, miR-151-5p and miR-222 were well correlated with clinicopathological variables such as nodal status and tumour size. Moreover, expression levels of miR-151-5p and miR-222 decreased after tumour excision. To the best of our knowledge, Yu *et al.* were the first to explore and

report serum miRNA levels in thyroid cancer patients by RNA sequencing. Obviously, the diagnostic potential requires further validation; however, this approach bypasses all the operator-dependent challenges of obtaining a sufficient amount of RNA from a single *in vivo* ultrasound-guided FNA and could be the blueprint for future minimally invasive diagnostic miRNA studies.

Studies of miRNAs in FTC have been reported by a limited number of research groups. At an early stage, Weber *et al.* (2006) examined miRNAs from primary FTC ($n=23$) and FA ($n=20$) tissue samples with microarray analysis. They found that miR-192, miR-346, miR-328 and miR-197 were overexpressed by 1.3- to 1.8-fold in FTC compared with FA. Functional studies showed that miR-346 and miR-197 induced proliferation in a non-thyroid cell line. Moreover, knockdown of miR-346 and miR-197 resulted in growth arrest in two follicular thyroid cancer cell lines but not in a papillary thyroid cancer cell line, indicating that miR-346 and miR-197 are specific in the pathogenesis of FTC. In addition, Nikiforova *et al.* (2008) examined nine FTCs and eight FAs using a RT-PCR miRNA panel consisting of 158 human miRNAs. They reported overexpression of miR-187, miR-181b, miR-182, miR-221, miR-222 and miR-96 in both FTC and oncocytic FTC in comparison to NTT, as well as overexpression of miR-181b, miR-339 and miR-224 in both conventional FA and oncocytic FA (Nikiforova *et al.* 2008). Both miR-200a and miR-181b were up-regulated in conventional FTC and FA. Comparison of the results of these two studies reveals very little conformity. This is not surprising given the studies used different quantification techniques (microarray vs RT-PCR) as well as different set-ups in the comparison of diagnostic groups (FTC/FA vs FTC/NTT).

A study conducted by our group analysed tissue samples from 12 FTCs, 12 FAs and ten NTTs using the Exiqon microarray platform containing probes for more than 800 human miRNAs. We confirmed some of the previously reported miRNAs as aberrantly expressed in FTC, namely up-regulation of miR-221, miR-222, miR-182 and miR-96 and also identified a number of miRNAs not previously identified in FTC (Rossing *et al.* 2012). A large portion of the down-regulated miRNAs was shared among the carcinomas and adenomas and the level of down-regulation did not differ much between the two tumour classes, indicating that the miRNA expression level changes represent an early event in follicular cell malignant transformation. A clear gradual down-regulation of miR-199b-5p was, however, detected from NTT over FA to FTC where it was completely absent. Moreover, functional analyses demonstrated miR-199b-5p

as an anti-proliferative component in follicular thyroid neoplasia. The numerous down-regulated miRNAs in FTC were explored by weighted target analyses, revealing a significant predominance of target transcripts encoding tumourigenic factors. This finding was also in concert with the mRNA profiles of our earlier study, which showed a significant up-regulation of these miRNA target transcripts (Borup *et al.* 2010). Furthermore, gene set enrichment analysis of our target transcripts showed an overlap with 'Rodrigues Thyroid Carcinoma Anaplastic Cluster', indicating a gradual pathogenic transformation of FTC to ATC. This association between FTC and ATC was supported by common features of our miRNA profiles and those reported in ATC by Braun *et al.* (2010).

The miRNA profile of ATC was first reported by Visone *et al.* (2007a), who compared ten ATC tissue samples with their adjacent tissues. They showed a clear over-representation of decreased miRNAs such as miR-30d, miR-125b, miR-26a and miR-30a-5p. Additionally, no miRNAs were up-regulated greater than twofold; however, miR-222 was 1.98-fold up-regulated. Validation was performed and functional experiments identified miR-125b and miR-26a as important regulators of cellular growth. The aforementioned study by Braun *et al.* (2010) not only confirmed the high frequency of under-expressed miRNAs in ATC, but also found that the miR-200 and miR-30 families clearly distinguished ATCs from PTCs and FTCs. In addition, functional studies showed that both the miR-200 and miR-30 families could decrease invasion of ATC-derived cells and hence these families were classified as 'anti-invasive miRNAs'. A global miRNA profile based on the ARO cell line was established by Takakura *et al.* (2008), who reported up-regulation of the miR-17-92 cluster and demonstrated growth reduction by selective inhibition of miR-17-3p, miR-17-5p and miR19-a.

ATC is a rare cancer and the studies by Braun *et al.* and Visone *et al.* are the only studies exploring the global miRNA profile based on eight or more tissue samples. For the rare thyroid cancers, it may be beneficial to merge the microarray data across studies. Consequently, 'noise' from different tissue types, RNA extraction methods, microarray platforms and variation in different array batches would be minimised and the results would include only the most consistent of the aberrantly expressed miRNAs.

The important regulatory function of miR-221/-222 by direct targeting of the well-characterised p27(Kip1) tumour suppressor transcript in a papillary thyroid cancer cell line, as well as other cancer cell lines, has been established (Galardi *et al.* 2007, Visone *et al.* 2007b). Subsequently, miR-221/-222 have been suggested as a

family of oncogenes, i.e. onco-miRNAs. It is likely that miR-221/-222 also function as oncogenes by decreasing p27 protein expression in FTC. Thus, a complete functional study in the relevant cell lines exploring this hypothesis requires to be conducted.

Nikiforova *et al.* (2008) reported a correlation between the miRNA expression pattern and thyroid cancer specimens with somatic mutations in *BRAF*, *RET/PTC* or *PAX8/PPAR (PPARG)* translocations. Their study identified five differentially expressed miRNAs – miR-146b, miR-155, miR-187, miR-221 and miR-222 – among the individual groups of mutations. This finding indicates that in addition to the marked changes in the miRNA expression profile between malignant and normal cells, the individual mutational status of the malignant cells is also of some significance. By using whole-genome sequencing of cancers, a recently launched database designated 'SomamiR' depicts somatic mutations located within miRNAs or their target sites (Bhattacharya *et al.* 2013). Somatic mutations that alter miRNA target sites have not yet been identified in follicular cell-derived thyroid cancer.

Building of molecular classification models

An approach for building and improving a molecular classification model based on tissue specimens from thyroid nodules starts with a set of good-quality RNA samples from snap-frozen tissue or FNA samples with concomitant histopathological diagnosis. Subsequent expression profiling employing high-throughput analysis is typically able to screen each sample for more than 50 000 probes, including redundant ones. Statistical analysis includes Student's *t*-test (adjusted *P* value to eliminate type 1 errors), probe ranking, model fitting and training by a suitable algorithm. It is noteworthy that discrimination is often obtained from the expression levels of only a small fraction (below a few per cent) of the number of transcripts analysed. Upon construction of an initial classifier from the expression profiles of the set of samples by the use of an appropriate algorithm, a validation of classifier performance is conducted by recording the diagnostic accuracy when applied to profiles from an independent set of samples (Simon 2003). It is possible to improve the original classifier by incorporation of additional samples. The classifier will of course be improved by increasing the number of samples as background noise will be reduced. Also, the histopathological diagnosis equal to the 'gold standard' can never be completely flawless and an increasing number of samples will minimise the noise from incorrectly

classified samples. Recently, a large study has stressed the importance of using the same type of tissue samples for the initial classifier construction as only moderate diagnostic accuracy was encountered when applying a tissue sample-derived classifier to FNA samples, whereas the performance of a classifier based on FNAs was markedly better (see next section; Chudova *et al.* 2010).

mRNA-based classification models

One of the remaining major challenges in the assessment of thyroid nodules is the differentiation between follicular cell-derived thyroid cancers and hyperplastic benign nodules based on preoperative FNAs. The diagnostic criteria for FTC rely on the presence of capsular and/or vascular invasion of tumour cells and this cannot be assessed from a cytological smear. Despite a clear histopathological definition of the malignancies of a given follicular neoplasia, the morphological findings are similar in FTC and FA. The molecular factors that determine a possible step-by-step malignant transition are still unresolved. Consequently, several studies have focused on the molecular classification of thyroid cancer and the benign counterparts. The classification studies are summarised in Table 1 and selected articles are discussed below.

Thomas Fahey and his collaborators were one of the first research groups to turn to gene expression profiling in an attempt to overcome the deficiencies of single marker analysis (Barden *et al.* 2003, Finley *et al.* 2004a,b, 2005). The studies were based on mRNA microarray analysis from a relatively small number of well-characterised snap-frozen tissue samples from FTC (minimally and widely invasive), Hürtle cell thyroid carcinoma (HTC), FA, HYP, FVPTC and PTC. The data analysis identified differentially expressed transcripts between the malignant and the benign lesions by comparing PTC vs HYP, FTC vs FA, and a pool of follicular cell-derived cancers vs FA and HYP together. Subsequently, a diagnostic sensitivity and specificity for detecting malignancy were calculated, and, as shown in Table 1, all the diagnostic accuracies from these studies were high. However, differentially expressed transcripts used to distinguish the different diagnostic groups were not filtered for their ability to discriminate, thus resulting in a high number of transcripts. Based on mRNA expression profiles, Weber *et al.* (2005) set out to find the smallest number of genes that could reliably classify FTC from FA, not just in the original data set comprising 41 follicular neoplasia, but ultimately in the validation samples ($n=31$). Following ranking of probes and testing of different combinations of gene sets,

the study concluded that FTCs could be discriminated from FA with a sensitivity of 100% and a specificity of 94.7% by accessing the expression values of cyclin D2 (*CCND2*), protein convertase 2 (*PCSK2*) and prostate differentiation factor (*PLAB/GDF15*). *CCND2* and *PCSK2* were highly up-regulated in FTC vs FA, and *PLAB* was fivefold down-regulated. A report by Jarzab *et al.* (2005) was one of the first that addressed the diagnostic implications of gene expression profiles by developing a molecular classifier comprising 19 transcripts (*DPP4*, *GJB3*, *ST14*, *SERPINA1*, *LRP4*, *MET*, *EVA1* (*MPZL2*), *SPUVE* (*PRSS23*), *LGALS3*, *HBB*, *MRKN2*, *MRC2*, *IGSF1*, *KIAA0830* (*ENDOD1*), *RXRG*, *P4HA2*, *CDH3*, *IL13RA1* and *MTMR4*). The biological relevance of the selected transcripts showed an over-representation of cell–cell adhesion genes, leading the authors to hypothesise that the cell adhesion genes could relate to the invasive and metastatic process of PTC. The classifier was based on the support vector machine algorithm, including leave-one-out cross-validation. The molecular signature of 19 transcripts could classify PTCs from benign nodules and NTT with a sensitivity and specificity of 85.7 and 100% respectively. Finally, Jarzab *et al.* tested the classifier on the available PTC gene expression profiles reported by Huang *et al.* (2001) and found that it was able to categorise NTT and PTC correctly. Eszlinger *et al.* (2006) contributed to this report by recommending the validation of every new molecular classifier across data sets derived from different array generations. A study by Prasad *et al.* (2008) investigated the potential of a molecular classifier for differentiating between malignant and benign thyroid nodules by including all the eight tumour subtypes that can be associated with suspicious cytology (HYP, FA, Hürtle cell adenoma, lymphatic nodules, PTC, FVPTC, FTC and Hürtle cell carcinoma) distributed over 94 fresh frozen tissue samples. Seventy-five transcripts were differentially expressed between the malignant and benign groups and using nearest-neighbour classification they obtained 73% sensitivity, an 82% specificity and a positive predicted value (PPV) of 78% for predicting malignancies. Validation of the expression levels of 12 genes (*HMG2*, *LRRK2*, *PLAG1*, *DPP4*, *CDH3*, *CEACAM6*, *PRSS3*, *SPOCK1*, *PDE5A*, *RAG2*, *AGTR1* and *TPO5*) selected based on their rank order and their apparent biological importance in tumour development and cancer progression were examined in an independent validation tumour set of 31 samples and the results were more or less consistent with the array data. No attempt to use this reduced gene set for classification was reported at that time. Nevertheless, a recent study from the same group explored the

diagnostic potential of a three-gene model; *HMGA2*, *MRC2* (mannose receptor, C type 2) and *SFN* (stratifin) (Prasad *et al.* 2012). Based on QRT-PCR data from 95 intra-operative-collected FNAs, Prasad *et al.* (2012) reported the ability of this three-gene model to correctly diagnose a FNA as malignant with a sensitivity of 71%, specificity of 84%, PPV of 65% and a negative predicted value (NPV) of 88%. The classification study performed by our group focused on developing a genetic signature that could distinguish between FTC and FA as well as FEA/micro-follicular adenoma (Borup *et al.* 2010). Upon microarray analysis of 92 tissue samples (83 follicular cell-derived malignant and benign plus nine NTTs), the comparative analysis identified 117, 240 and 512 differentially expressed probe sets between FTC and FA, FEA and FA, and FTC and FEA. In each leave-one-out loop, probes were ranked as well as fitted by the support vector machine algorithm. Following this principle, three classifiers were generated. Furthermore, a predicted probability was assigned to each sample upon classification. The main classifier (classifier 1 consisting of 76 probe sets) was generated to separate FTC from FA and did so with high accuracy (sensitivity=94.4%, specificity=95.5%, PPV=94.4% and NPV=95.5%). Moreover, the robustness of the classifier was validated by employing publicly available data as well as cross-platform data. The classifier provided the ability to diagnose follicular nodules originating from different geographical locations and platforms with similar accuracy.

The large multicentre study by Chudova *et al.* (2010) built two diagnostic classifiers by implementing the support vector machine algorithm on a large set of fresh frozen tissue samples ($n=178$) and *in vivo* and *ex vivo* FNAs ($n=137$) and subsequently evaluating the performance in an independent set of FNAs ($n=48$). Testing of the tissue-trained classifier on the independent FNAs showed a sensitivity and specificity of 92 and 58% respectively for classifying malignancies. The relatively poor specificity could be due to fundamental differences in the biological or technical properties of tissue samples and FNAs. Thus, the classifier trained on the FNA samples diagnosed malignant tumours with a specificity of 84% and a NPV of 96%. Furthermore, the study explored the impact of diluting the expression values of their signatures with benign tissue. *In silico* and *in vitro* studies showed that the derived classifier was able to classify malignancies in most cases in the presence of 80% benign background. The project has been ongoing and the classifier subsequently evaluated in a series of indeterminate FNAs obtained from 49 USA academic and community-based sites, with

Alexander *et al.* (2012) presenting its performance. The final classifier was based on 167 transcripts selected by their performance ranking in a run on 32 remaining samples after excluding 31 FNAs for various reasons. Subsequently, the selected transcripts were measured in 233 new samples remaining from 328 after similar exclusions, and classifier performance was tested. The combined set of 265 samples comprised a total of ten cases of FTC. Unfortunately, 14% of the tested samples with an acceptable RNA quality were excluded post-unblinding due to shipment deviation. In these final remaining samples, the classifier identified malignancy with a sensitivity and specificity of 92 and 52% respectively. This is not a sufficient specificity for decision making, i.e. malignant or benign, immediate surgery or follow-up. The NPV was 93%, which is promising when the aim is to rule out malignancy. Moreover, the classifier showed high NPVs for selected subgroups of indeterminate nodules (95% for atypical or follicular lesion of undetermined significance, 94% for follicular neoplasm or lesion suggestive of follicular neoplasm and 85% for a suspicious cytological finding). The data indicate that by measuring the expression values of the 167 transcripts from a cytologically indeterminate FNA (based on a minimum of two needle insertions), it might be possible to consider a more conservative approach for patients with 'benign' as classifier result. The classifier consists of transcripts encoding proteins involved in a variety of cellular processes, the most frequent being related to energy metabolism, cell differentiation and cellular development, indicating shifts in energy-producing pathways, commonly observed in cancer cells and exemplified by the Warburg effect (Kim & Dang 2006).

Concerning the classifier suggested by Alexander *et al.*, a subsequent study authored by many of the same researchers tested the analytic performance of the classifier in diagnosing cytologically indeterminate FNAs (Walsh *et al.* 2012). No significant differences regarding sample pre-processing as well as diagnostic sensitivity and sensitivity of the classifier were found upon comparison of the results obtained in the two different studies. These results may also emphasise the importance of experienced operators when performing high-throughput molecular analyses.

Comparing the diagnostic performances in different studies addressing the main diagnostic challenge of follicular neoplasia, i.e. the discrimination between FTC and FA (Table 1), it appears that quantification of a relatively high number (greater than about 100) of transcripts is required to rule out malignancies with a high certainty (NPV > 95%). This may reflect the heterogeneous expression pattern characteristics of FTC.

miRNA-based classification models

Mazeh *et al.* (2011) examined the expression levels of miR-21, miR-31, miR-146b, miR-187, miR-221 and miR-222 in *ex vivo* FNAs (FNA performed in the operating room after tumour resection) from PTCs ($n=20$) and HYPs ($n=7$). The evaluated miRNAs were known to be altered in PTC but their diagnostic potential had not been examined previously. The results showed that miR-146b, miR-221 and miR-222 could individually differentiate between PTC and HYP with NPVs $>70\%$ as shown in Table 2. The results did not comprise a merging of the expression levels of all the evaluated miRNAs, which may have improved the diagnostic potential. Along the same vein, a comprehensive study by Vriens *et al.* (2012) encompassing 95 tissue samples (12 FTCs, 15 FAs, 12 Hürtle cell adenomas, 12 Hürtle cell carcinomas, 8 PTCs, 4 ATCs, 12 FVPTCs, 13 HYPs and 7 NTTs) and more than 120 *ex vivo* FNAs identified several differentially expressed miRNAs by comparing the malignant and benign neoplasia

(miR-100, miR-125b, miR-138 and miR-768-3p were up-regulated in the malignant entities). Subsequent QRT-PCR analysis of the specific miRNAs revealed miR-138 to be differentially expressed between benign and malignant FNAs ($P<0.001$) and an ultimate diagnostic accuracy of 75% and NPV of 81%.

The miRNA signatures for classification of follicular neoplasia developed in our own laboratory consisted of data derived from two global RNA platforms: a conventional spotted microarray platform (>800 annotated miRNAs plus >400 proprietary miR-E-Plus sequences) and newly developed Universal RT miRNA PCR panels I and II enabling profiling of 742 human miRNAs (Rossing *et al.* 2012). The microarray classifier comprised just two miRNAs, miR-1826 and miR-E-Plus-1078, and diagnosed FTC with a PPV and NPV of 83%, whereas the QRT-PCR-derived classifier comprised 14 miRNAs and diagnosed FTC with a PPV of 100% and a NPV of 92%. Recently, Shen *et al.* (2012) examined a panel of miRNAs by QRT-PCR in RNA extracted from archived FNA specimens

Table 2 Studies of miRNA-based classification

Type of tumours	Sample type	Method	MicroRNA	Diagnostic classes	Diagnostic performance ^a	References
20 PTC, 7 HYP	<i>Ex vivo</i> FNAs	QRT-PCR	miR-21, -31, -146b, -187, -221, -222	PTC vs HYP	miR-221: Sens. = 95% Spec. = 100% PPV = 100% NPV = 96%	Mazeh <i>et al.</i> (2011)
12 FTC, 12 FA, 10 NTT	34 fresh frozen tissues	Microarray QRT-PCR panel	Microarray: miR-1826 and miR-E-Plus-1078 QRT-PCR panel: miR-19a, -501-3p, -17, -335, -106b, -15a, -16, -374a, -542-5p, -503, -320a, -326, -330-5p, let-7i	FTC vs FA	Microarray: PPV = 83% NPV = 83% QRT-PCR panel: PPV = 100% NPV = 92%	Rossing <i>et al.</i> (2012)
88 malignant (PTC, FTC, FVPTC, ATC), 40 benign (HYP, HTA, FA)	128 archived FNAs	QRT-PCR	miR-146b, -221, -187, -30d	Malignant vs benign	Training set: PPV = 98% NPV = 83% Validation set: PPV = 89% NPV = 78% Undetermined/atypia cytology: PPV = 64% NPV = 79%	Shen <i>et al.</i> (2012)
10 FVPTC, 35 PTC, 111 benign (HYP, HTA, FA)	95 intraoperative FNAs + validation set ($n=59$)	QRT-PCR	miR-7	Malignant vs benign	Sens. = 100% Spec. = 29% PPV = 36% NPV = 100%	Kitano <i>et al.</i> (2012)

HTA, Hürtle cell thyroid adenoma; HA, Hürtle cell adenoma; Sens., sensitivity; Spec., specificity.

^aDiagnostic accuracy for predicting malignancies.

($n=128$) and by linear discriminant analysis established a four-miRNA signature for diagnosing FNA with undetermined cytology. As detailed in Table 2, the performance of the classifier in the undetermined FNA samples leaves room for further refinement. However, the comprehensive results of extracting miRNA from archived FNAs could prove to have a major impact on future thyroid FNA classification studies. Recently, Kitano *et al.* (2012) reported that miR-7 could be used as an adjunct marker for malignancy based on their QRT-PCR analysis of intraoperative FNAs. The study evaluated four different miRNAs, miR-7, miR-126, miR-374a and let-7g in the training set and found only miR-7 to be significantly down-regulated in the malignant samples. Subsequently, the miR-7 expression level was used to diagnose malignancy in the validation set and achieved a good result regarding the ability to rule out malignancy (NPV = 100%). However, the PPV and specificity were low, emphasizing the need for a broader signature preferably comprising both up-regulated and down-regulated miRNAs.

Final remarks

The main focus of the studies analysing samples of follicular neoplasia has been classification of tumours with indeterminate cytology. However, a consistent molecular profile has not been established for FTC. This could be due to true biological heterogeneity and/or reflect the difficulties in diagnosing follicular neoplasia correctly. Identification of a molecular signature is indeed dependent on the correctness of the initial histopathological diagnoses of the tumour samples on which the classifier is built. The fact that the focus of the studies has been on the differentiation between FTC and FA may also have hindered the establishment of a possible consistent molecular profile of FTC. This is in contrast to the easier diagnosed PTC, where a more uniform molecular profile has been identified and shows, for example, overexpression of *FN1*, *MET*, *CITED1*, *SERPINA1*, miR-146b, miR-21, miR-221 and miR-222.

One way of achieving the inherent profile of follicular cell-derived cancers might be by performing a comprehensive study – a large number of fresh frozen FTC specimens, unambiguously classified and with known mutational status. To diminish the contamination of NTT, it may be beneficial to supplement the analyses with RNA extracted from well-defined laser-captured microdissected FTC tumour tissue. Laser capture microdissection has the capacity to collect representative mRNA patterns in cancer and normal cells, leaving all unwanted cells behind. In this

way, it is possible to separate sets of cells from normal, precancerous and cancerous tissue and potentially obtain a more characteristic molecular profile for the heterogenic group of follicular neoplasia represented by FTC and FA diagnoses. This scenario may follow the procedure established by Leethanakul *et al.* (2000) concerning the application of laser capture microdissection to head and neck squamous cell carcinomas.

Furthermore, upcoming next-generation sequencing-based studies may identify new mutations characterizing the different variants of follicular cell-derived cancer. This technique examines mutational status at the level of both RNA and DNA, the latter being appealing as DNA is stable and can be easily obtained from FNAs in a clinical setting. Based on the recent progress in molecular diagnostic procedures and the advent of the next-generation sequencing technique, it seems realistic that molecular classification will improve the current evaluation of cold thyroid nodules in the near future.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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