Trefoil Factor 3 (TFF3): A Promising Indicator for Diagnosing Thyroid Follicular Carcinoma

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Abstract. Since the introduction of fine needle aspiration biopsy (FNAB) in the 1970’s, a preoperative diagnostic technique for thyroid follicular carcinoma has long been awaited. Many markers that distinguish follicular carcinomas from adenomas have been reported; however, most of them have not been confirmed to be beneficial for clinical use. Trefoil factor 3 (TFF3) is a relatively new family of peptides that bears the three-loop trefoil domain. Several groups have reported that the suppression of TFF3 mRNA expression is related to malignant characteristics of thyroid follicular cell-derived tumors and the expression level of TFF3 mRNA is the most promising indicator for diagnosing follicular carcinoma. Development of TFF3-based diagnostic methods is now ongoing and it may not be long before thyroid follicular carcinoma can be diagnosed preoperatively using an aspirated sample from the tumor.

Keywords: Thyroid follicular carcinoma, Trefoil factor 3, Aspiration biopsy cytology, Gene expression

Preoperative diagnosis of thyroid follicular carcinoma: a clinical test that has been awaited for 30 years

Thyroid nodules are relatively common, especially in women [1]. Recently, because of the wide use of ultrasonography for screening of thyroid tumors or arteriosclerotic disease, the number of patients recognized to have occult small thyroid nodules has increased, and clinical management of these patients has engendered a new controversy [2, 3].

There are three major types of thyroid cancer believed to be derived from thyroid follicular cells: papillary carcinoma, anaplastic carcinomas and follicular carcinomas. Follicular thyroid carcinoma is a relatively uncommon malignancy and accounts for about 15% of all thyroid cancers [4]. Pathologically, follicular carcinomas are defined as malignant neoplasms of the thyroid epithelium that exhibit follicular differentiation. Histologically, they are characterized by varying degrees of resemblance to normal follicular architecture and function, including colloid formation and capsule formation. They show signs of capsular invasion, as well as local tissue and vascular invasiveness. They are classified into two groups, a minimally invasive type and a widely invasive type. The former is much more common and closely resembles benign follicular adenoma in its gross and microscopic appearance. One of the most difficult distinctions in thyroid pathology is the differentiation between benign follicular adenomas which account for over 90% of thyroid tumors, and minimally invasive follicular carcinomas. The principal differentiating feature is capsular or vascular invasion. Even this, however, may not be definitive, because slight capsular penetration can also be observed in benign tumors. Further, both types of tumors have varying degrees of cellular atypia and extensive invasion into vascular spaces is not usually observed in minimally invasive carcinomas [5]. Since the introduction of fine needle aspiration biopsy (FNAB) in the early 1970’s, diagnosis of the majority of thyroid malignancies, especially papillary carcino-

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ma, can be made without much difficulty [6]. However, preoperative diagnosis of follicular carcinoma still remains difficult since capsular or vascular invasion is not possible to determine cytologically. Most patients who have been diagnosed with thyroid follicular tumors require follow-up examinations for the rest of their life. When the tumors can not be diagnosed by cytological examinations, identification of a definite molecular marker is crucial for establishment of a preoperative test to diagnose follicular carcinoma. Thus, such a marker has been awaited for more than 30 years and has been searched by many researchers.

Search for molecular markers that differentiate follicular carcinoma from adenoma

Since the 1980s, cancer cells, including those of the thyroid, have been considered to be derived from well-differentiated normal cells such as thyroid follicular cells (thyrocytes) via multiple incidents of genomic damage especially to oncogenes or anti-oncogenes, that accelerate proliferation or foster malignant phenotypes, such as the ability to invade surrounding tissue or metastasize to distant organs. In light of these considerations, many researchers have searched for specific genomic alternations in follicular carcinomas [7, 8].

The PAX8-PPARγ1 gene fusion has been identified in a significant subset of follicular carcinomas. This rearrangement leads to an in-frame fusion of the PAX8 gene, which encodes a paired domain transcription factor, with the peroxisome proliferator-activated receptor (PPAR) γ1 gene. In the first original report, PAX8-PPARγ1 fusion was detected in 62.5% of follicular carcinomas, but not in follicular adenomas, papillary carcinomas, or adenomatous goiters, suggesting that it may be a specific molecular marker for follicular carcinoma [9]. However, in recent follow-up studies, PAX8-PPARγ1 was identified in 26-56% of follicular carcinomas and in up to 13% of follicular adenomas, thus it is now considered that PAX8-PPARγ1 is detectable in both follicular adenomas and carcinomas, but not in papillary or anaplastic carcinomas [10, 11].

Another strategy is to find mRNAs or proteins that are specifically expressed in follicular carcinoma. MET encodes a transmembrane tyrosine kinase identified as the receptor of hepatocyte growth factor (HGF). One report originally described overexpression of MET mRNA in papillary and follicular carcinoma [12]. A series of follow-up studies confirmed its overexpression in papillary carcinomas. On the other hand, the increased expression of MET mRNA in follicular carcinomas compared to follicular adenomas has not been clearly demonstrated, although some studies with DNA microarrays showed significant differences [13].

Telomerase is an enzyme that elongates the chromosomal end or telomere. Its catalytic component, human telomerase reverse transcriptase (hTERT), is known to be expressed in immortalized cell lines and human carcinomas, but not in most normal differentiated cells. Some reports have described possible differentiation of follicular carcinomas from adenomas, even using aspirated samples, by reverse transcription-polymerase chain reaction (RT-PCR) detection of hTERT mRNA [14-16]. However, some recent studies failed to confirm the utility of this technique. These findings were explained partly by the expression of hTERT mRNA in contaminated lymphocytes [17, 18].

Bartolazzi et al. reported a striking result in 2001. Using immunohistochemical analysis of galectin-3 (LGALS3), correct diagnoses of follicular carcinoma were made in 99% and 92% of cases when using histological and cytological samples, respectively [19]. Galectin-3 is a β-galactosyl-binding protein involved in regulating cell-cell and cell-matrix interactions. Its increased expression has been observed in both papillary carcinomas and follicular carcinomas, but not in follicular adenomas, adenomatous goiters, or normal thyroid tissues. However, the results of the follow-up studies are quite controversial. Most of recent studies have revealed two main findings. First, efficient diagnosis of papillary carcinomas can be made by immunohistochemical analysis of galectin-3 as described in the first report, but follicular carcinomas cannot be diagnosed [20–22]. Second, there is no significant difference in the expression of LGALS3 mRNA between follicular carcinomas and follicular adenomas [23, 24].

Very recently, three studies concerning molecular markers of follicular carcinoma were published. Using serial analysis of gene expression (SAGE), Cerutti et al. identified four differentially expressed genes (DDIT3, ARG2, ITM1, C1orf24) between follicular adenomas and carcinomas and confirmed the results by immunohistochemistry [25]. Further, using DNA micro-arrays, Barden et al. identified ADM, BSG, and
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ENPP2 in 2003, and Weber et al. identified CCND2, PCSK2 and PLAB in 2005 [13, 26]. However, the utility and reproducibility of these results have not yet been confirmed in detail. These markers are summarized in Table 1.

Trefoil Factor 3 (TFF3) as the best indicator for diagnosing follicular carcinoma

TFF3 is a relatively new family of peptides that bear the three-loop trefoil domain [27]. They are mainly synthesized and secreted by mucin-secreting epithelial cells lining the gastrointestinal tract and have a close association with mucins. They are highly conserved during evolution and are resistant to heat, acid, and enzymes. The function of TFF peptides is still unknown; however, abundant expression in various ulcerative conditions suggests an important role in mucosal defense and repair. Even less is known about TFF3, since it was the last to be identified. TFF3 is expressed mainly in goblet cells of the small and large intestine and has recently been abundantly found in salivary glands [28, 29]. Differential expression of TFF3 mRNA between follicular adenomas and carcinomas was first reported in detail in our study using SAGE in 2004 [30]. We estimated the efficiency of differential diagnosis between follicular adenoma and carcinoma by measuring the expression levels of TFF3 mRNA. In the 54 follicular adenomas and 29 follicular carcinomas analyzed, the relative expression of TFF3 mRNA was markedly decreased in 7 follicular carcinomas of the widely invasive type and with evident distant metastases. When the appropriate cutoff point was set and pathologically questionable cases were excluded, the sensitivity, specificity and accuracy were 80.0%, 91.5%, and 87.5%, respectively [31]. This first report did not attract much attention from researchers probably because of the following two reasons. First, TFF3 mRNA is down-regulated in thyroid malignancies, weakening the impact of TFF3 mRNA as a marker since it is generally recognized that tumor markers are overexpressed in malignancies. Second, the overall accuracy of TFF3 mRNA for diagnosis of thyroid follicular carcinoma is about 80 to 90%, which was regarded to be quite low because other researchers reported some molecular makers that showed perfect concordance with pathological diagnoses.

However, while other markers failed to show reproducible results in follow-up studies, researchers at other institutes revealed the utility of TFF3 mRNA for the diagnosis of follicular carcinoma [32–35]. In 2005, Taniguchi et al. used a unique system for analysis of gene expression called Adapter-Tagged Competitive PCR, to screen over 2000 genes expressed in thyroid follicular tumors and concluded that TFF3 mRNA is one of the most promising markers for diagnosing follicular carcinoma [32]. In 2007, Foukakis et al. reevaluated 26 previously reported candidate genes and found that the combination of TFF3 and hTERT mRNA could most efficiently distinguish follicular carcinomas from adenomas [33]. Another re-evaluation had performed by Krause et al. in 2008 [34], who examined 10 candidate genes and found that downregulation of TFF3 in malignant tumors was the best indicator for thyroid malignancies including follicular

<table>
<thead>
<tr>
<th>Gene</th>
<th>reference</th>
<th>sensitivity</th>
<th>specificity</th>
<th>accuracy</th>
<th>results in follow-up studies* [references]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX8-PPARY</td>
<td>9</td>
<td>62.5%</td>
<td>100%</td>
<td>89.2%</td>
<td>D [10, 11]</td>
</tr>
<tr>
<td>MET</td>
<td>12</td>
<td>22.2%</td>
<td>100%</td>
<td>65%</td>
<td>B [13]</td>
</tr>
<tr>
<td>hTERT</td>
<td>15</td>
<td>100%</td>
<td>71.4%</td>
<td>84.6%</td>
<td>B [17, 18]</td>
</tr>
<tr>
<td>LGALS3</td>
<td>19</td>
<td>100%</td>
<td>98%</td>
<td>99%</td>
<td>D [20–24]</td>
</tr>
<tr>
<td>DDIT3</td>
<td>25</td>
<td>85.1%</td>
<td>90.6%</td>
<td>88.1%</td>
<td>C</td>
</tr>
<tr>
<td>ARG2</td>
<td>25</td>
<td>85.1%</td>
<td>90.6%</td>
<td>88.1%</td>
<td>C</td>
</tr>
<tr>
<td>ADM, BSG, ENPP</td>
<td>13</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>C [34]</td>
</tr>
<tr>
<td>CCND2, PCSK2, PLAB</td>
<td>26</td>
<td>100%</td>
<td>94.7%</td>
<td>96.7%</td>
<td>C [34]</td>
</tr>
<tr>
<td>TFF3</td>
<td>31</td>
<td>72.4%</td>
<td>83.3%</td>
<td>79.5%</td>
<td>A [32–35]</td>
</tr>
</tbody>
</table>

*A, reproducibility has been confirmed in several studies; B, reproducibility has been confirmed only in a few studies, probably because the expression level of the target gene does not show great difference; C, utility of the target gene has not been confirmed in detail; D, the follow-up studies showed conflicting results with the first study.
carcinoma and any combination assays that include TFF3 could efficiently distinguish follicular carcinomas from adenomas.

**Discord between molecular-based and pathological diagnoses of thyroid follicular carcinoma**

Upregulation of TFF3 is observed in normal thyroid tissues, adenomatous goiters, 80% of follicular adenomas, and 20% of minimally invasive follicular carcinomas, while downregulation is observed in anaplastic carcinomas, papillary carcinomas, widely invasive follicular carcinomas, 80% of minimally invasive follicular carcinomas, and 20% of follicular adenomas [30, 33, 34] (Fig. 1). Thus, TFF3 mRNA is a universal indicator for thyroid tumors derived from thyroid follicular cells.

Some groups reported that combinations of several target genes showed almost perfect concordance with pathological diagnoses [26, 36]. However, this combination analysis has not been validated because each group presented a completely different set of genes as the most useful one and their results have not been confirmed by other groups. From a pathological point of view, the results in some previous studies of molecular-based diagnosis of thyroid follicular carcinoma are far from convincing; although these studies included a considerable number of minimally invasive carcinomas, the investigators still correctly diagnosed nearly all cases. The diagnosis of minimally invasive follicular carcinomas is very difficult, even with pathological examination [5]. Thus, a perfect concordance between pathological diagnosis and molecular-based diagnosis is difficult to believe. Downregulation of TFF3 is observed in all follicular tumors that can be definitely diagnosed as malignant such as widely invasive follicular carcinomas and minimally invasive carcinomas with distant metastases [30, 33]. Considering these facts, the discrepancy between pathological and TFF3-based diagnosis may be at least in part due to the difficulty in diagnosis of minimally invasive follicular carcinoma. Taniguchi *et al.* provided evidence to support this hypothesis [32]. They chose 60 genes that were differentially expressed between follicular adenomas and carcinomas, and searched for combinations of genes that could diagnose follicular carcinoma with high accuracy. Accuracy only reached 90% even when using all 60 genes. Thus, perfect concordance between pathological and molecular-based diagnoses can never happen, at least when we use the gene expression profile as an indicator. When pathologically questionable cases were excluded, the diagnostic accuracy of TFF3-based diagnosis reached 87.5%, which is almost the same as Taniguchi’s combination assay [31]. In preoperative diagnosis, accurate quantitative measurement of multiple mRNAs is quite difficult since only a small number of tumor cells can be obtained by FNAB. Thus, it should be carefully discussed if there are any evident advantages of combination assays over measuring TFF3 expression alone.

Thyroid tumors derived from follicular cells including follicular tumors, may be classified into two groups: benign follicular tumors with upregulated TFF3 expression, and possibly malignant follicular tumors with downregulated TFF3 expression. Only the latter may be indicated for surgical resection (Table 2).

**Advantages of TFF3 as an indicator of thyroid malignancy**

Up to several hundreds of cells containing 10 ng of total RNA are usually aspirated by FNAB [37]. Thus, since high sensitivity preoperative tests for diagnosing follicular carcinoma is required; only abundantly expressed genes can be targeted with preoperative diagnostic methods. TFF3 mRNA is expressed abundantly in benign thyroid tumors. Its expression level is 10 to 100 times greater than that of beta-actin (ACTB).
mRNA, and almost as much as that of thyroglobulin (TG) mRNA, which shows the most abundant expression in thyroid cells [30, 38]. On the contrary, most of genes reported to be useful for diagnosing follicular carcinoma show relatively low expression levels. For example, the expression level of hTERT mRNA, which was reported as one of the most promising genes for diagnosing follicular carcinoma, is 10,000-fold less than that of ACTB mRNA [39]. Thus, it is difficult to carry out the quantitative measurement of these genes in aspirated samples.

Another aspect to be considered in the establishment of preoperative diagnostic methods is the sensitivity of the assay. Certain genes or certain sets of genes show higher diagnostic accuracy than TFF3. However, when considering the “preoperative” use of the test, sensitivity is the most important factor. For example, Foukakis et al. proposed a set of two genes, hTERT and TFF3, as a diagnostic indicator [33]. While this set efficiently differentiated follicular adenomas and carcinomas, it diagnosed 2 of 13 obviously malignant cases, which include widely invasive follicular carcinomas and minimally invasive follicular carcinomas with distant metastases as “benign”. Thus, this set is not suitable as a screening test for malignancy. In our and Foukakis’s reports, down regulation of TFF3 is observed in all cases of widely invasive follicular carcinoma and minimally invasive follicular carcinoma with distant metastases, which are regarded as obviously malignant [30, 31, 33]. Thus, TFF3 is reliable indicator for preoperative screening of thyroid malignancy since it does not miss “true” malignant cases.

### Measuring TFF3 expression in aspirates from thyroid tumors

In order to establish a molecular-based preoperative test for follicular carcinoma, precise measurement of TFF3 expression is crucial. Although the easiest method of examining protein expression in cells is immunohistochemistry, immunohistochemical examination of TFF3 protein expression in thyroid tumor cells has not been reported to date. Although unclear, following two reasons have been speculated. First, TFF3 mRNA is abundantly expressed even in follicular carcinoma and difference in the expression levels between adenomas and carcinomas ranges between 10 to 10²-fold [30, 34]. Thus, a clear difference in immunological staining may not be observed using anti-TFF3 antibodies. Second, since the majority of TFF3 protein is secreted by cells, the precise estimation of

### Table 2. Classification of thyroid follicular tumors by TFF3 mRNA expression

<table>
<thead>
<tr>
<th>TFF3 mRNA</th>
<th>pathological classification</th>
<th>% of tumors*</th>
</tr>
</thead>
<tbody>
<tr>
<td>overexpressed tumor</td>
<td>follicular adenoma</td>
<td>84%</td>
</tr>
<tr>
<td></td>
<td>minimally invasive follicular carcinoma</td>
<td>29%</td>
</tr>
<tr>
<td>underexpressed tumor</td>
<td>follicular adenoma</td>
<td>16%</td>
</tr>
<tr>
<td></td>
<td>minimally invasive follicular carcinoma</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td>minimally invasive follicular carcinoma with distant metastases</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>widely invasive follicular carcinoma</td>
<td>100%</td>
</tr>
</tbody>
</table>

*These data were cited from reference 30.

**Why does the loss of TFF3 expression relate to malignant characteristics of thyroid tumors?**

Follicular carcinomas are believed to be derived from follicular adenomas by multi-step carcinogenesis, in which damage to the genome of a follicular adenoma cell results in the rise of a malignant follicular carcinoma cell [8]. Thus, TFF3 may be regarded as tumor suppressor gene, since loss of expression is observed in malignant thyroid tumors. However, tumor suppressor role of TFF3 has not been demonstrated in functional studies to date and while TFF3 is downregulated in colon cancer, TFF3 mRNA is overexpressed in prostate and esophageal cancers [40–42].

Molecular-based diagnosis of anaplastic and papillary carcinomas can be established easily with genes overexpressed restrictedly in these tumors in clear contrast to follicular carcinoma [43–45]. The reason why TFF3 is downregulated in follicular carcinomas is not clear at present. The further investigation to clarify this phenomenon may give a clue to understand the very fundamental mechanism of thyroid carcinogenesis.
expression levels by cytological immunohistochemistry may be difficult [27].

Measurement of the expression level of TFF3 mRNA in aspirates is also problematic because of interference by contaminating peripheral blood cells or infiltrating lymphocytes. The expression level of TFF3 mRNA in aspirates does not always correlate with that in the corresponding tumor tissue [46]. We reported a method to separate thyroid tumor cells from blood cells using mesh filtration (Fig. 2) [46]. After mesh filtration, the expression level of TFF3 mRNA in aspirates correlated well with that of corresponding tumor tissues. This is probably because thyroid follicular tumor cells tend to become attached to each other and are usually aspirated as a cluster rather than a single cell, thus blood cells but not follicular tumor cells can pass through the mesh.

Another possible method to measure TFF3 mRNA expression in aspirates in the near future is quantum-dot (Q-dot) RNA in situ hybridization (Q-RISH) [47, 48]. A Q-dot is a fluorescent semiconductor nanocrystal that possesses extremely high fluorescence efficiency and photostability, making it near-optimal for many fluorescent applications. Q-dots have recently been used for molecular and cellular labeling and more recently for in situ hybridization. TFF3 mRNA was easily detected by non-radioisotopic in situ hybridization due to its abundant expression [30]. Using Q-RISH, we may be able to not only identify the localization of TFF3 mRNA but also estimate the expression level of TFF3 mRNA in each cell in the aspirate.

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References


