Diagnostic and prognostic \textit{TERT} promoter mutations in thyroid fine-needle aspiration biopsy

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Abstract
Two promoter mutations, chr5:1 295 228C\textsuperscript{O}T and chr5:1 295 250C\textsuperscript{O}T, in the gene for telomerase reverse transcriptase (TERT) have been recently identified in thyroid cancers and shown to be important in thyroid tumor pathogenesis. The diagnostic and prognostic potentials of testing for these mutations on thyroid fine-needle aspiration biopsy (FNAB) have not been investigated. Herein, we examined the two TERT promoter mutations along with the \textit{BRAF} V600E mutation by direct DNA sequencing on 308 FNAB specimens preoperatively obtained from thyroid nodules with postoperatively confirmed pathological diagnoses. We found TERT promoter mutations in 0.0% (0/179) of benign thyroid nodules and 7.0% (9/129) of thyroid nodules of differentiated thyroid cancer, representing a 100% diagnostic specificity and 7.0% sensitivity, with the latter rising to 38.0% (49/129) when combined with \textit{BRAF} V600E testing. Several TERT-promoter-mutation-positive thyroid nodules were cytologically indeterminate on FNAB. Approximately 80% of the TERT promoter mutation-positive thyroid nodules were thyroid cancers with aggressive clinicopathological behaviors, such as extrathyroidal invasion, lymph node metastases, distant metastases, disease recurrence or patient death. Thus, a positive TERT promoter mutation test not only definitively diagnoses a thyroid nodule as cancerous but also preoperatively identifies a cancer with aggressive potential. This is the first study, to our knowledge, of TERT promoter mutations on thyroid FNAB, demonstrating the value of this novel molecular testing in the diagnosis of thyroid nodules and preoperative risk stratification of thyroid cancer. Thus, testing of TERT promoter mutations on FNAB will enhance and improve the current molecular-based approaches to the management of thyroid nodules and thyroid cancer.

Introduction
Thyroid cancer is a common endocrine malignancy, which has seen a worldwide rapid rise in incidence in recent years (Jemal \textit{et al.} 2011, Howlader \textit{et al.} 2014). In the USA, there are 62,980 new cases of thyroid cancer and 1890 deaths from this cancer estimated for 2014 (Howlader \textit{et al.} 2014). The diagnosis of thyroid cancer typically starts from the evaluation of thyroid nodules, which are extremely common, seen in approximately 5–10% of adult people on physical examination and 50–70% of people over the age of 60 years on ultrasonography (Mazzaferrri 1993, Guth \textit{et al.} 2009). Clinical evaluation of thyroid nodules for malignancy is therefore
a major task in the practice of thyroid medicine, in which a diagnostic mainstay is fine-needle aspiration biopsy (FNAB). FNAB is accurate in most patients in that it can provide a reliable diagnosis of benign or malignant thyroid tumor (Bose & Walts 2012). In approximately 25–30% of cases, however, FNAB yields indeterminate cytological findings, leaving the diagnosis of thyroid nodules in this group in dilemma. With an overall risk of approximately 25% for malignancy associated with the indeterminate cytology on FNAB, patients in this group are conventionally recommended for thyroidectomy and, as a result, most of these patients have their thyroid glands destroyed as a consequence of benign thyroid tumors (Cooper et al. 2009). Along with this diagnostic challenge of thyroid nodules, there are also prognostic issues associated with thyroid cancer. Although thyroid cancer in most patients is indolent with an excellent prognosis, some cases seem to be destined for poor prognosis with increased disease recurrence and patient mortality. Risk stratification for prognostication of thyroid cancer has been conventionally based on clinicopathological risk factors, which are often inaccurate and preoperatively unavailable.

In recent years, molecularly based diagnostic and prognostic approaches for thyroid cancer have been extensively investigated and some molecular markers have been identified and proven to be clinically useful (Xing et al. 2013a). These include diagnostically the gene expression classifier (Alexander et al. 2012), genetic marker panel (Nikiforov et al. 2011), and galectin 3 (Bartolazzi et al. 2008) and prognostically BRAF V600E mutation (Xing et al. 2013b, 2014a). However, the diagnostic and prognostic accuracies of these markers still have much room for improvement and new markers are required to achieve this.

Two recently discovered mutations in the promoter of the gene for telomerase reverse transcriptase (TERT) in thyroid cancer show promise in this regard – chr5:1295228C>T and chr5:1295250C>T (termed herein as C228T and C250T respectively), which represent C>T nucleotide changes at positions −124 and −146 from the ATG translation start site of the TERT gene respectively. These mutations increase the transcriptional activities of the TERT promoter (Horn et al. 2013, Huang et al. 2013). As such, this discovery has important implications for human cancers as TERT has been known to play an important role in cellular immortality by maintaining the telomere length at the end of chromosomes and in promoting other cellular functions such as proliferation and cell cycles (Smekalova et al. 2012, Mocellin et al. 2013). Our group reported the first study, to our knowledge, on these two mutations in thyroid cancer (Liu et al. 2013a), in which we found a prevalence of mutations of 0.0% (0/85), 11.7% (30/257), 13.9% (11/79), 37.5% (3/8), and 46.3% (25/54) in benign thyroid tumors, papillary thyroid cancers (PTCs), follicular thyroid cancers (FTCs), poorly differentiated thyroid cancers, and anaplastic thyroid cancers (ATCs) respectively. This study also revealed an exclusive occurrence of TERT promoter mutations in thyroid cancer but not in benign thyroid tumors and also their association with poor clinicopathological characteristics of thyroid cancer. These findings were confirmed in our and others’ subsequent studies (Liu et al. 2013b, 2014, Vinagre et al. 2013, Melo et al. 2014). We have recently also demonstrated that TERT promoter and BRAF V600E mutations cooperatively identify the most aggressive PTCs with the highest recurrence (Xing et al. 2014b). These results strongly indicate that TERT promoter mutations are potential novel diagnostic and prognostic biomarkers for thyroid cancer. Herein, we performed the first study, to our knowledge, to directly evaluate the value of preoperatively testing TERT promoter mutations of thyroid FNAB specimens in the diagnostic evaluation of thyroid nodules and preoperative prognostic evaluation of thyroid cancer.

Materials and methods

FNAB samples and DNA preparation

A total of 308 FNAB specimens were obtained preoperatively by FNAB to thyroid nodules in 308 patients who underwent thyroidectomy for established thyroid cancer, cytologically indeterminate thyroid nodules, or symptomatic goiter as described previously (Xing et al. 2004, 2009). Genomic DNA from FNAB specimens was isolated using standard procedures of protease K digestion, phenol–chloroform extraction, and ethanol precipitation. This study was conducted based on the protocol approved by an institutional review board and written informed consents were obtained from patients where appropriate.

Genomic DNA sequencing to identify TERT promoter and BRAF V600E mutations

Standard PCR was performed for direct genomic DNA sequencing to identify TERT promoter mutations as we described previously (Liu et al. 2013a). Briefly, a fragment of the TERT promoter, which contained the sites for TERT promoter mutations C228T and C250T, was amplified by PCR on genomic DNA from FNAB specimens using primers 5’-AGTGGATTCGCGGGCACAGA-3’ (sense) and 5’-CAGCGCCTGCTGAAACTC-3’ (antisense). PCR of standard reaction mixture containing approximately 40–50 ng
genomic DNA/reaction was performed with initial denaturation at 95 °C for 3 min, followed by ten cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 68 °C for 1 min. This was then followed by 30 cycles of the same settings except for the elongation for an additional 5 s in each cycle and completion with elongation at 68 °C for 7 min. Quality confirmation of the PCR products was achieved by gel electrophoresis and sequencing PCR was performed using a Big Dye terminator v3.1 cycle sequencing reaction kit (Applied Biosystems) and an ABI PRISM 3730 automated next generation genetic analyzer (Applied Biosystems) at our institutional sequencing facility.

Identification of the BRAF V600E mutation was similarly achieved by direct genomic DNA sequencing, as described previously (Xing et al. 2005). Briefly, PCR was performed to amplify exon 15 of the BRAF gene containing the site for the T1799A (V600E) mutation using primers TCATAATGCTTGCTCTGATAGGA (sense) and GGCCAAAATTTAATCAGTGGA (antisense). This resulted in a 212-bp PCR product. The PCR parameters used were as follows: one cycle of 95 °C for 5 min; two cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; two cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, followed by extension at 72 °C for 5 min. After quality confirmation by gel electrophoresis, the PCR products were subjected to Big Dye reaction and sequencing analysis.

Results

Diagnostic potential of testing thyroid FNAB specimens for TERT promoter mutations

We analyzed the status of the two TERT promoter mutations and one BRAF V600E mutation for 308 FNAB specimens obtained preoperatively from 308 patients with confirmed postoperative pathological diagnosis of the biopsied thyroid nodules. These included 111 PTCs, 18 FTCs, and 179 benign thyroid nodules (including 111 cases of adenomas, 55 cases of multinodular hyperplasia, and 13 cases of Hashimoto’s thyroiditis). As is shown in Table 1, no TERT promoter mutation was found in any of the 179 benign thyroid nodules. TERT promoter mutations C228T and C250T were found in nine cases of thyroid cancers, including five PTCs and four FTCs, with a collective prevalence of 7.0% (9/129) in thyroid cancers, which was slightly lower than the reported prevalence in these cancers (Liu et al. 2013a,b, 2014, Vinagre et al. 2013, Melo et al. 2014), probably reflecting the compromised detection sensitivity of direct genetic sequencing on FNAB specimens, which often contain sparse cancer cells as addressed previously (Xing et al. 2004). The BRAF V600E mutation was found solely in thyroid nodules of PTCs, the frequency being 37.8% (42/111), which was slightly lower than the prevalence of this mutation generally observed in PTCs (Xing 2005, 2007), again reflecting an underdetection by direct genetic sequencing on FNAB specimens (Xing et al. 2004). There were two cases of BRAF V600E-positive PTCs that additionally harbored TERT promoter mutations, with one harboring TERT C228T and the other harboring TERT C250T. If any mutation was counted, the collective prevalence of TERT promoter and BRAF V600E mutations was 40.5% (45/111) in thyroid nodules of PTCs. When thyroid nodules of PTCs and FTCs were collectively analyzed, BRAF V600E was found in 32.6% (42/129) and TERT promoter and BRAF mutations were collectively found in 38.0% (49/129) of the cases. Based on these results, the diagnostic specificity of TERT promoter mutations in FNAB specimens for thyroid cancer was 100% and the sensitivity was 7.0%. When TERT promoter mutations were used in combination with BRAF V600E, the diagnostic specificity remained 100% and the sensitivity rose to 38.0%. This also represented an increase from the diagnostic sensitivity of 32.6% when BRAF mutation alone was used (Table 1). Three cases of the TERT promoter

<table>
<thead>
<tr>
<th>Thyroid conditions</th>
<th>TERT C228T mutation, n/N (%)</th>
<th>TERT C250T mutation, n/N (%)</th>
<th>Two TERT mutations collectively</th>
<th>BRAF V600E mutation, n/N (%)</th>
<th>Any mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign thyroid nodulesa</td>
<td>0/179 (0)</td>
<td>0/179 (0)</td>
<td></td>
<td>0/179 (0)</td>
<td>0/179 (0)</td>
</tr>
<tr>
<td>PTC</td>
<td>4/111 (3.6)</td>
<td>1/111 (0.9)</td>
<td>5/111 (4.5)</td>
<td>42/111 (37.8)</td>
<td>45/111 (40.5)</td>
</tr>
<tr>
<td>FTC</td>
<td>3/18 (16.7)</td>
<td>1/18 (5.6)</td>
<td>4/18 (22.2)</td>
<td>0/18 (0)</td>
<td>4/18 (22.2)</td>
</tr>
<tr>
<td>PTC + FTC</td>
<td>7/129 (5.4)</td>
<td>2/129 (1.6)</td>
<td>9/129 (7.0)</td>
<td>42/129 (32.6)</td>
<td>49/129 (38.0)</td>
</tr>
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PTC, papillary thyroid cancer; FTC, follicular thyroid cancer.
*aOne case of PTC harbored both TERT C228T and BRAF V600E mutations and another harbored both TERT C250T and BRAF V600E mutations.
*aBenign thyroid nodules included 111 cases of adenomas, 55 cases of multinodular hyperplasia, and 13 cases of Hashimoto’s thyroiditis.
mutation-positive thyroid nodules of FTCs showed indeterminate cytological findings on FNAB. Thus, preoperative testing for TERT promoter mutations on FNAB could help make a definitive diagnosis of thyroid cancer in these cases of thyroid nodules.

**Prognostic potential of preoperatively testing thyroid FNAB specimens for TERT promoter mutations**

Mostly, the TERT-promoter-mutation-positive thyroid nodules were thyroid cancers that exhibited aggressive clinicopathological outcomes, such as lymph node metastases, extrathyroidal invasion, distant metastases, tumor recurrence, or even patient death. Out of five patients with TERT-promoter-mutation-positive thyroid nodules of PTCs, four had such outcomes. Specifically, patient 1 (MX225), positive on preoperative FNAB for TERT C228T, was a 51-year-old man, in whom thyroidectomy revealed a 2.0-cm PTC in the right thyroid lobe with extrathyroidal invasion and metastases in 20 out of 85 neck lymph nodes. Patient 2 (MX249), positive on preoperative FNAB for TERT C228T, was also a 51-year-old man, in whom thyroidectomy revealed a 3.5 cm tumor with mixed PTCs and ATCs in the left thyroid lobe with extrathyroidal invasion and metastases in 11 out of 18 lymph nodes.

Post surgery imaging showed extensive metastases in the lungs. He died 1 year after the initial diagnosis. Patient 3 (MX279), positive on preoperative FNAB for both the TERT C228T and BRAF V600E mutations, was a 54-year-old man, in whom thyroidectomy revealed a 2.5-cm PTC with tall cell component in the right thyroid lobe, with metastases in seven out of 16 lymph nodes and invasion to trachea, requiring tracheotomy. He had metastatic recurrence to the right posterior ilium and the lungs at the follow-up of 31 months after the initial treatments, which were radiodine non-avid. Patient 4 (MX466), positive on preoperative FNAB both for TERT C250T and BRAF V600E, was a 74-year-old man, in whom thyroidectomy revealed multifocal PTCs with the largest being 3.0 cm in the left thyroid lobe with vascular invasion and metastases in four out of 11 lymph nodes. Even with radiodine ablation after thyroidectomy, thyrotropin-stimulated thyroglobulin level increased to 3.0 ng/ml at 38 months after the initial treatments. The patient was undergoing further diagnostic evaluations at the time of this writing. Patient 5 (MX525), positive for the TERT C228T mutation on preoperative FNAB, was a 47-year-old man, in whom thyroidectomy revealed a 2.5-cm PTC in the right thyroid lobe without lymph node removal. He has continued to do well with no apparent disease recurrence at 55 months after the initial treatments. The two cases of patients with coexisting TERT promoter and BRAF V600E mutations both had disease recurrence.

Out of four patients with TERT-promoter-mutation-positive thyroid nodules of FTCs, three exhibited poor clinicopathological outcomes. Specifically, patient 1 (MX39), positive on preoperative FNAB for TERT C228T, was a 54-year-old woman, in whom thyroidectomy revealed a worrisome large FTC of 6.5 cm and she is currently being clinically followed. Patient 2 (MX66), positive on preoperative FNAB for TERT C250T, was a 77-year-old man, in whom thyroidectomy revealed a 8.0-cm FTC in the left thyroid lobe, with gradually rising serum thyroglobulin in the subsequent years, and a 6.0-cm recurrent FTC in the left neck as well as lung metastases were found 11 years after the initial treatments. Patient 3 (MX238), positive for the TERT C228T mutation on preoperative FNAB, was a 74-year-old man, in whom thyroidectomy revealed a 5.0-cm FTC in the right thyroid lobe with extensive extrathyroidal and vascular invasion.

A post-radiiodine-therapy body scan showed wide bony metastasis to clivus in the skull base, sternum, proximal right upper extremity, right proximal humerus, thoracic and lumbar spine, a left posterior inferior rib, right scapula, pelvis, and bilateral femurs. The patient died from extensive FTC metastases 10 months after the initial diagnosis and treatments. Patient 4 (MX488), positive on preoperative FNAB for TERT C250T, was a 67-year-old woman, in whom thyroidectomy revealed a 1.5-cm FTC in the left thyroid lobe with no recurrence at 32 months of follow-up after the initial treatments.

Overall, seven out of nine (78%) thyroid cancer patients who were TERT-promoter-mutation-positive on preoperative FNAB testing of the thyroid nodules exhibited aggressive tumor behaviors and poor clinical outcomes, including disease recurrence and patient deaths in several cases. This represents a poorer prognosis than generally observed with PTCs and FTCs.

**Discussion**

This is the first study, to our knowledge, directly investigating the diagnostic and prognostic potentials of preoperative testing of thyroid FNAB specimens for the recently discovered TERT promoter mutations in thyroid cancer. The prevalence of TERT promoter mutations in differentiated thyroid cancer (PTCs and FTCs) found in this study was lower than the generally reported prevalence in primary tumors, reflecting an expected underestimation on mutation testing by direct DNA sequencing of FNAB.
specimens, sometimes due to spare cancer cells (Xing et al. 2004). This test sensitivity can be expected to be improved using more sensitive testing modalities, such as the Mutecor colorimetric assay (Xing et al. 2004, 2009) or the real-time Light Cycler PCR and fluorescence melting curve analysis (Nikiforov et al. 2011). Nevertheless, this study principally demonstrates the feasibility of testing TERT promoter mutations on routine FNAB specimens. Compared with the BRAF V600E mutation, the prevalence of TERT promoter mutations is relatively low in thyroid cancer. Thus, the diagnostic sensitivity of TERT promoter mutation testing alone on FNAB is low. However, the sensitivity could be increased when TERT promoter mutations are used in combination with other diagnostic molecular markers. This may be true particularly when more sensitive testing methods are used and TERT promoter mutations are tested in conjunction with the currently known molecular markers, such as BRAF mutation, RAS mutation, and RET/PTC and PAX8/PPARγ (PPARG) rearrangements, which had a sensitivity of approximately 90% for thyroid nodules of indeterminate cytology on FNAB (Nikiforov et al. 2011). The BRAF mutation has an established diagnostic and prognostic utility for thyroid cancer when detected on FNAB specimens (Xing et al. 2004, 2009, Mekel et al. 2010). This study demonstrated that addition of TERT promoter mutations could increase the diagnostic sensitivity of the BRAF V600E mutation for thyroid cancer and was helpful in making a definitive diagnosis of thyroid cancer in some cases of cytologically indeterminate thyroid nodules. Thus, it might be expected that inclusion of TERT promoter mutations would improve the diagnostic sensitivity of the currently used panel of diagnostic genetic molecular markers for thyroid cancer, probably bringing the sensitivity to above 90%. It is also possible that addition of TERT promoter mutations may improve the diagnostic values of the gene expression classifier (Alexander et al. 2012) and galectin 3 (Bartolazzi et al. 2008). Importantly, in a large number of benign FNAB specimens, we found no TERT promoter mutation, consistent with the similar findings in primary tumors in several recent studies (Liu et al. 2013a, 2014, Vinagre et al. 2013, Melo et al. 2014), thus demonstrating a 100% diagnostic specificity. This means that a positive TERT promoter mutation test result on FNAB yields a definitive diagnosis of thyroid cancer. Therefore, testing of TERT promoter mutations on FNAB, particularly when used in conjunction with testing of the currently established molecular markers, will most probably have a useful diagnostic value that may improve the current diagnostic evaluation of thyroid nodules.

Several studies have demonstrated an association of TERT promoter mutations with aggressive clinicopathological characteristics (Liu et al. 2013a,b, 2014, Melo et al. 2014, Xing et al. 2014b). Consistent with these studies on primary tumors, this study demonstrated that TERT-promoter-mutation-positive thyroid nodules were not only detected in 100% of malignant tumors, but that these cancers mostly also behaved aggressively. Out of nine TERT-promoter-mutation-positive thyroid nodules, seven (78%) turned out to be aggressive cancers with multiple aggressive clinicopathological behaviors, including lymph node metastasis, extrathyroidal and local invasion, distant metastasis, tumor recurrence or patient deaths. In a cohort of 507 cases of PTC patients, we have recently demonstrated that coexistence of TERT promoter and BRAF mutations was associated with particularly aggressive clinicopathological outcomes of PTCs, including a dramatically increased recurrence risk (Xing et al. 2014b). Consistent with these findings, this study found two such cases of PTCs with dual mutations, both of which had aggressive tumor behaviors and disease recurrence. The lower rate of coexisting TERT promoter and BRAF mutations found in this study again probably reflects the relatively low sensitivity of direct genetic sequencing of FNAB specimens (Xing et al. 2004). The present results of directly testing for TERT promoter mutations using FNAB specimens provide the first direct evidence, to our knowledge, demonstrating the prognostic potential of preoperatively testing for these mutations for thyroid cancer – a positive result for the TERT promoter mutation predicts preoperatively poorer clinicopathological outcomes of thyroid cancer. Thus, such a positive preoperative TERT promoter mutation test result would favor more aggressive treatments of the patient, such as more aggressive initial thyroid surgery and subsequent more vigilant monitoring for disease recurrence.

In summary, this is the first study, to our knowledge, of preoperatively testing, for TERT promoter mutations along with BRAF V600E on FNAB, demonstrating the strong diagnostic and prognostic potential of this novel molecular tests for thyroid cancer. The results provide important evidence supporting the inclusion of TERT promoter mutations in the currently used thyroid molecular tests to assist with the diagnosis of thyroid nodules and preoperative risk stratification for better management of thyroid cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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