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Diagnostic and Prognostic *TERT* Promoter Mutations in Thyroid Fine Needle Aspiration Biopsy

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Abstract

Two promoter mutations, chr5:1,295,228C>T and chr5:1,295,250C>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 these mutations on thyroid fine needle aspiration biopsy (FNAB) have not been investigated. Here, we examined the two *TERT* promoter mutations along with the *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 *BRAF* V600E testing. Several *TERT* promoter mutation-positive thyroid nodules were cytologically indeterminate on FNAB. Nearly 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 cancer but also preoperatively identifies a cancer with aggressive potential. This is the first study of *TERT* promoter mutations on thyroid FNAB, demonstrating the value of this novel molecular testing in the diagnosis of thyroid nodule 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 nodule and thyroid cancer.

Keywords

T*ERT* promoter mutation; thyroid cancer; fine needle aspiration biopsy; *BRAF* V600E mutation; telomerase reverse transcriptase

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Introduction

Thyroid cancer is a common endocrine malignancy, which has seen a worldwide rapid rise in incidence in recent years (Howlader *et al*. 2014; Jemal *et al*. 2011). In the United States, there are 62,980 new cases of thyroid cancer and 1,890 deaths from this cancer estimated for 2014 (Howlader *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 (Guth *et al*. 2009; Mazzaferri 1993). 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 about 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 about 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 to sacrifice their thyroid glands for benign thyroid tumors (Cooper *et al*. 2009). Along with this diagnostic challenge of thyroid nodules, there are also prognostic issues 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, molecular-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 galctin-3 (Bartolazzi *et al*. 2008) and prognostically *BRAF* V600E mutation (Xing *et al*. 2014a; Xing *et al*. 2013b). The diagnostic and prognostic accuracy of these markers, however, still have much room for improvement and new markers are needed to this end.

Two recently discovered mutations in the promoter of the gene for telomerase reverse transcriptase (TERT) in thyroid cancer provide promises in this regard—chr5:1,295,228C>T and chr5:1,295,250C>T (termed here as C228T and C250T, respectively), which represent nucleotide changes of-124 C>T and −146 C>T 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 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 on these two mutations in thyroid cancer (Liu X *et al*. 2013), 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 (PTC), follicular thyroid cancer (FTC), poorly differentiated thyroid cancers, and anaplastic thyroid cancer (ATC), respectively.

This study also demonstrated 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 X *et al*. 2014; Melo *et al*. 2014;Vinagre *et al*. 2013; Liu T *et al*. 2013). We have recently also demonstrated that *TERT* promoter and *BRAF* V600E mutations cooperatively identify the most aggressive PTC with the highest recurrence (Xing *et al*. 2014b). These studies strongly suggest that *TERT* promoter mutations are potential novel diagnostic and prognostic biomarkers for thyroid cancer. Here, we performed the first study to directly evaluate the value of preoperatively testing *TERT* promoter mutations on 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

Three hundreds and eight 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*. 2009; Xing *et al*. 2004). Genomic DNA from FNAB specimens was isolated using standard procedures of protease K digestion, phenol–chloroform extraction, and ethanol precipitation. The study was conducted based on an institutional review board-approved protocol and written informed patient consents were obtained where appropriate.

Genomic DNA sequencing to identify TERT promoter and BRAF V600E mutations

Standard polymerase chain reaction (PCR) was performed for direct genomic DNA sequencing to identify *TERT* promoter mutations as we described previously (Liu X *et al*. 2013). 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' CAGCGCTGCCTGAAACTC 3'(antisense). PCR of standard reaction mixture containing about 40–50 ng genomic DNA/reaction was performed with an initial denaturation at 95°C for 3 min, followed by 10 cycles of 95°C denaturation for 30 seconds, 55°C annealing for 30 seconds, and 68°C elongation for 1 min. This was then followed by 30 cycles of the same settings except for the elongation for additional 5 seconds in each cycle and the completion with an 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 GGCCAAAAATTTAATCAGTGGA (antisense). This resulted in a 212-bp PCR product.

The PCR settings included 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; 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by an 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 *BRAF* V600E mutation on 308 FNAB specimens obtained preoperatively from 308 patients with confirmed postoperative pathological diagnosis of the biopsied thyroid nodules. These included 111 PTC, 18 FTC, and 179 benign thyroid nodules (including 111 cases of adenomas, 55 cases of multinodular hyperplasia, and 13cases of Hashimoto's thyroiditis). As 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 9 cases of thyroid cancers, including 5 PTC and 4 FTC, 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*. 2013; Liu *et al*. 2014; Melo *et al*. 2014; Vinagre *et al*. 2013), likely 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 PTC, being 37.8% (42/111), which was slightly lower than the prevalence of this mutation generally seen in PTC (Xing 2005; Xing 2007), again reflecting an under detection by direct genetic sequencing on FNAB specimens (Xing *et al*. 2004). There were two cases of *BRAF* V600E-positive PTC 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 PTC. When thyroid nodules of PTC and FTC 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 on FANB specimens for thyroid cancer was 100% and sensitivity 7.0%. When *TERT* promoter mutations were used in combination with *BRAF* V600E, the diagnostic specificity remained to be 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 mutation-positive thyroid nodules of FTC 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

The *TERT* promoter mutation-positive thyroid nodules mostly were thyroid cancers that exhibited aggressive clinicopathological outcomes, such as lymph node metastases, extrathyroidal invasion, distant metastases, tumor recurrence or even patient death. Four of

the five patients with *TERT* promoter mutation-positive thyroid nodules of PTC 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 20/85 neck lymph nodes. Patient 2 (MX 249), 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 PTC and ATC in the left thyroid lobe with extrathyroidal invasion and metastases in 11 of 18 lymph nodes. Post-surgery imaging showed extensive metastases in the lungs. He died one 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 7/16 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 radioiodine 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 PTC with the largest being 3.0 cm in the left thyroid lobe with vascular invasion and metastases in 4 of 11 lymph nodes. Even with radioiodine ablation after thyroidectomy, thyrotropin-stimulated thyroglobulin rose to 3.0 ng/mL 38 months after the initial treatments, which was undergoing further diagnostic evaluations at the time of this writing. Patient 5 (MX525), positive for *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 continued to be doing well with no apparent disease recurrence 55 months after the initial treatments. The two cases of patients with coexisting *TERT* promoter and *BRAF* V600E mutations both had disease recurrence.

Three of the four patients with *TERT* promoter mutation-positive thyroid nodules of FTC 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 clinically followed. Patient 2 (MX66), positive on preoperative FNAB for *TERT* C250T, was a 77-year-old man, in whom thyroidectomy reveled 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 lungs metastases were found 11 years after the initial treatments. Patient 3 (MX238), positive for *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. Post-radioiodine 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 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 seen with PTC and FTC.

Discussion

This is the first study directly investigating the diagnostic and prognostic potentials of preoperatively testing on thyroid FNAB specimens for the recently discovered *TERT* promoter mutations in thyroid cancer. The prevalence of *TERT* promoter mutations in differentiated thyroid cancer (PTC and FTC) found in the present study was lower than the generally reported prevalence in primary tumors, reflecting an expected underestimate on mutation testing by direct DNA sequencing on FNAB specimens due to spare cancer cells sometimes (Xing *et al*. 2004). This test sensitivity can be expected to be improved by using more sensitive testing modalities, such as the Mutector colorimetric assay (Xing *et al*. 2009; Xing *et al*. 2004) or the real-time Light Cycler PCR and fluorescence melting curve analysis (Nikiforov *et al*. 2011). Nevertheless, the present 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 FANB is low. The sensitivity, however, 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* rearrangements, which had a sensitivity of close to 90% for thyroid nodules of indeterminate cytology on FNAB (Nikiforov *et al*. 2011). *BRAF* mutation has an established diagnostic and prognostic utility for thyroid cancer when detected on FNAB specimens (Xing *et al*. 2004; Xing *et al*. 2009; Mekel *et al*. 2010). The present study demonstrated that addition of *TERT* promoter mutations could increase the diagnostic sensitivity of *BRAF* V600E mutation for thyroid cancer and were helpful in making a definitive diagnosis of thyroid cancer in some cases of cytologically indeterminate thyroid nodules. Thus, it is expectable that inclusion of *TERT* promoter mutations would improve the diagnostic sensitivity of the currently used panel of diagnostic genetic molecular markers for thyroid cancer, likely brining 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 X *et al*. 2013; Liu X *et al*. 2014; Melo *et al*. 2014; Vinagre *et al*. 2013), thus demonstrating a 100% diagnostic specificity. This means that a positive *TERT* promoter mutation test result on FNAB makes a definitive diagnosis of thyroid cancer. Therefore, testing of *TERT* promoter mutations on FANB, particularly when used in conjunction with testing of the currently established molecular markers, will most likely have a useful diagnostic value that may improve the current diagnostic evaluation of thyroid nodules.

Several studies demonstrated an association of *TERT* promoter mutations with aggressive clinicopathological characteristics (Liu X *et al*. 2013; Liu X *et al*. 2014; Melo *et al*. 2014; Xing *et al*. 2014b; Liu T *et al*. 2013). Consistent with these studies on primary tumors, the

present study demonstrated that *TERT* promoter mutation-positive thyroid nodules were not only 100% malignant tumors but these cancers mostly also behaved aggressively. Seven of the nine (78%) *TERT* promoter mutation-positive thyroid nodules 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 recently demonstrated that coexistence of *TERT* promoter and *BRAF* mutations was associated with particularly aggressive clinicopathological outcomes of PTC, including a dramatically increased recurrence risk (Xing *et al*. 2014b). To be consistent with these findings, the present study found two such cases of PTC 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 the present study again likely reflects the relatively low sensitivity of direct genetic sequencing on FNAB specimens (Xing *et al*. 2004). The present results on directly testing *TERT* promoter mutations on FANB specimens provide the first direct evidence demonstrating the prognostic potential of preoperatively testing these mutations for thyroid cancer— a positive result of *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 of preoperatively testing *TERT* promoter mutations along with *BRAF* V600E on FNAB, demonstrating strong diagnostic and prognostic potentials of this novel molecular test for thyroid cancer. The results provide important evidence supporting the inclusion of *TERT* promoter mutations in the currently used thyroid molecular testing to assist the diagnosis of thyroid nodules and preoperative risk stratification for better management of thyroid cancer.

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Table 1

Testing for *TERT* promoter and *BRAF* V600E mutations on thyroid fine needle aspiration biopsy

Thyroid conditions	TERT C228T mutation, n/N (%)	TERT C250T mutation, n/N (%)	Two TERT mutations collectively	BRAF V600E mutation, n/N (%)	Any mutation**
Benign thyroid nodules*	0/179(0)	0/179(0)	0/179(0)	0/179(0)	0/179(0)
PTC	4/111(3.6)	1/111(0.9)	5/111(4.5)	42/111(37.8)	45/111(40.5)
FTC	3/18(16.7)	1/18(5.6)	4/18(22.2)	0/18(0)	4/18(22.2)
PTC+FTC	7/129(5.4)	2/129(1.6)	9/129(7.0)	42/129(32.6)	49/129(38.0)

*Benign thyroid nodules included 111cases of adenomas, 55 cases of multinodular hyperplasia, and 13cases of Hashimoto's thyroiditis.

**One case of PTC harbored both *TERT* C228T and *BRAF* V600E mutations and another harbored both *TERT* C250T and *BRAF* V600E mutations. PTC, papillary thyroid cancer; FTC, follicular thyroid cancer.