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Assessment of molecular testing in fine-needle aspiration biopsy samples: An experience in a Chinese population

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ABSTRACT

Fine-needle aspiration biopsy remains the mainstay for preoperative examination of thyroid nodules; however, it does not provide a definite diagnosis in up to 25% of nodules. Considerable studies have been performed to identify molecular markers to resolve this diagnostic dilemma. The aim of this study was to establish the distribution and frequency of common genetic alterations in a comprehensive set of benign and malignant thyroid nodules, and to determine the feasibility and role of testing for a panel of genetic alterations in improving the accuracy of cytology diagnosis in a Chinese population. This study was conducted in 314 thyroid nodules comprising 104 papillary thyroid carcinomas, 13 suspicious nodules, 52 indeterminate nodules, and 145 benign nodules. Point mutations and *RET/PTC* rearrangements, were evaluated by pyrosequencing and TaqMan real-time PCR, respectively. After surgery, 115 nodules were confirmed as conventional papillary thyroid carcinoma and 102 (88.70%) of these nodules harbored either the $BRAF^{V600E}$ mutation (76.52%) or *RET/PTC* rearrangements (12.17%). *RAS* mutation was found in 1 (33.33%) follicular thyroid carcinoma, 1 (14.29%) follicular thyroid adenoma and 4 (10%) goiter nodules. With cytology and molecular testing, the diagnostic accuracy was further increased to 98.82% in papillary thyroid carcinoma diagnosis, and was preoperatively increased to 76.92% and 84.00%, respectively, in nodules with suspicious and indeterminate cytology. In conclusion, molecular testing of a panel of genetic alterations in fine-needle aspiration biopsy can be effectively performed in clinical practice. It enhances the accuracy of cytology and is of particular value for indeterminate nodules in the Chinese population.

Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence is increasing in different countries (Dal Maso et al., 2011; Davies and Welch, 2014). In clinical practice, the majority of thyroid cancers manifest as thyroid nodules, which occur in 20 to 76% of adults. Approximately 5 to 15% of thyroid nodules were finally proved to be malignant by surgery (Popoveniuc and Jonklaas, 2012; Yassa et al., 2007). Fine-needle aspiration biopsy (FNAB) has emerged as the most reliable clinical tool for distinguishing malignant from benign nodules. However, nearly 10 to 25% of FNABs yield an indeterminate result with a risk of malignancy from 5 to 75% depending on the indeterminate category according to the Bethesda System for Reporting Thyroid Cytology (Bongiovanni et al., 2012). This group of indeterminate nodules is one of the greatest challenges to clinician in thyroid nodule management.

Various genetic alterations have been described in thyroid cancer during the last two decades, and molecular markers of thyroid cancer have been used in clinical practice to refine fine needle aspiration diagnosis of thyroid nodules (Gomez Saez, 2010; Nikiforova and Nikiforov, 2009). Genetic mutations including the $BRAF^{V600E}$ mutation, *RAS* point mutation and *RET/PTC* rearrangement have been widely studied in papillary and follicular thyroid carcinoma, which are the two most common thyroid cancers (~90 to 95%) originating from the thyroid follicular epithelium (Nikiforov and Nikiforova, 2011). *BRAF* is a serine–threonine kinase of the RAF family of proteins and the $BRAF^{V600E}$ mutation is the most common *BRAF* point mutation (Xing et al., 2004). The V600E mutation constitutively activates the *BRAF* kinase, leading to excessive stimulation of the mitogen-activated protein kinase (MAPK) pathway and ultimately, thyroid tumorigenesis (Kimura et al., 2003). $BRAF^{V600E}$ is the most common molecular alteration in papillary thyroid carcinoma (PTC) and is also detected in poorly differentiated and anaplastic thyroid carcinomas derived from *BRAF*-mutated PTCs (Nikiforova et al., 2003a). This point mutation has not been found in follicular thyroid carcinomas (FTC) or benign nodules, which suggests that it is specific for papillary thyroid carcinoma and related tumors. The frequency of the

Abbreviations: FNAB, fine-needle aspiration biopsy; MAPK, mitogen-activated protein kinase; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; FISH, fluorescence in situ hybridization; PI3K, phosphatidylinositol 3-kinase; FVPTC, follicular variant of papillary thyroid carcinoma; PCR, polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value; HCC, Hürthle cell carcinoma; FTA, follicular thyroid adenoma; HCA, Hürthle cell adenoma.

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BRAF^{V600E} mutation in PTCs was largely different with a range of ~40–80% in different studies (Lassalle et al., 2010). This difference in the reported prevalence of the *BRAF*^{V600E} mutation may be affected by factors, including age, techniques, geographic region, and histological subtype of the PTC (Xing, 2013). A recent study demonstrated that the prevalence of the *BRAF*^{V600E} mutation increased from 50% to 77% within classic PTC since 1974 at one US institution (Jung et al., 2014).

The RET/PTC chromosomal rearrangements could activate the *RET* proto-oncogene encoding a cell membrane receptor tyrosine kinase and then activate the MAPK signaling pathway (Pierotti et al., 1992; Santoro et al., 1994). *RET/PTC* activation is hypothesized to be oncogenic for thyroid follicular cells, because it transforms cells in vitro and results in the formation of thyroid tumors in transgenic mice (Jhiang, 2000; Jhiang et al., 1996; Santoro et al., 1996). *RET/PTC1* and *RET/PTC3* rearrangements are the two most common types of rearrangements in thyroid carcinoma (Fenton et al., 2000). The prevalence of the *RET/PTC* rearrangement in reported studies of thyroid tumors varies greatly. This variance may be due to geographic variability, varying sensitivities of methods and heterogeneous distribution of the *RET/PTC* rearrangement in tumors (Pacini et al., 2000). In tumors associated with radiation exposure, the *RET/PTC* chromosomal rearrangement was the most frequent genetic alteration during the development of tumorigenesis, particularly in children and young adults (Nikiforov, 2002). The diagnostic impact of the *RET/PTC* rearrangement has been evaluated in cytological specimens, in which its presence offers utility for specimens of indeterminate of morphology (Cheung et al., 2001; Salvatore et al., 2004). With indeterminate cytology, a positive *RET/PTC* rearrangement finding may lead to a PTC diagnosis. However, the specificity of *RET/PTC* for PTC has been challenged by several observations that reported its detection in benign thyroid follicular adenomas, benign and malignant oncocytic (Hürthle cell) tumors, and even in some non-neoplastic conditions such as Hashimoto's disease (Nikiforov, 2006). Most of those studies used a highly sensitive technique for analysis, which is capable of detecting a very small proportion of cells carrying a particular genetic event. Recently, a study demonstrated that fluorescence in situ hybridization (FISH) (Caria et al., 2013), the most sensitive method for detecting gene rearrangements in a single cell, could be used to distinguish nodules with abnormal follicular morpholoy with a ≥3% FISH signal for broken *RET*. When samples with a 6.8% break-apart FISH signal could be detected by RT-PCR and were proved to be malignant. In addition, the specificity of diagnosis was decreased from 100% by RT-PCR to 80% by FISH. In all, using appropriate detection methods, *RET/PTC* rearrangements are reasonably specific for papillary thyroid carcinoma.

The *RAS* gene encodes a family of three highly homologous isoforms: *HRAS*, *NRAS*, and *KRAS*. *RAS* activity is tightly regulated by GTP-mediated hydrolysis of activated GTP-bound *RAS* to inactivated GDP-bound *RAS* (Prior et al., 2012). A *RAS* point mutation exhibits either increased affinity for GTP (codons 12 and 13) or inhibition of the autocatalytic GTP-ase function (codon 61), resulting in constitutive aberrant activation of MAPK and the phosphatidylinositol 3-kinase (PI3k) signaling pathways (Fukushima and Takenoshita, 2005; Howell et al., 2013). Of all *RAS* mutations, 99% target either codons 12, 13 or 61 with variable preference and 95% of all *NRAS* mutations affect codon 61 (Prior et al., 2012). Previous studies reported that the *NRAS61* mutation was the most common, accounting for 52.6%–88% of *RAS* mutations (Bhaijee and Nikiforov, 2011; Fukushima and Takenoshita, 2005; Howell et al., 2013; Jang et al., 2014). Together with the *KRAS* mutation, these activating point mutations are very common in human cancers (S.J. Park et al., 2013). Otherwise, a previous study indicated that *NRAS61* was significantly associated with the presence of distant metastasis in FTC (Jang et al., 2014). Yet, unlike the *BRAF*^{V600E} mutation and *RET/PTC* rearrangements, which nearly are suggestive of a malignant diagnosis, the *RAS* mutation has been detected in the full spectrum of thyroid neoplasms ranging from benign follicular adenoma to anaplastic thyroid carcinoma (Esapa et al., 1999; Oyama et al., 1995; Vasko et al., 2003). Most studies have reported that the *RAS* mutation was preferentially associated with follicular patterned thyroid lesions, which always are difficult to differentiate as benign or malignant based on cytology, and are often indeterminate by FNAB (Gupta et al., 2013; J.Y. Park et al., 2013). The predominance of the *RAS* mutation in a follicular variant of PTC (FVPTC) and FTC indicated that *RAS* mutation testing in FNAB samples may be the most clinically useful (Lee et al., 2013; Zhu et al., 2003). In addition, there is a prevailing notion that *RAS*-positive follicular adenoma is likely a precursor lesion for the *RAS*-mediated development of follicular-patterned cancer (Gupta et al., 2013; Howell et al., 2013; Vasko et al., 2004). Taken together, although the identification of the *RAS* mutation in FNAB samples is not 100% predictive of cancer, it is highly suggestive of either FTC or FVPTC in indeterminate cytology.

These represent the most common genetic alterations in thyroid cancer, as well as molecular markers of diagnostic and prognostic significance. The number of studies on the impact of molecular diagnostic in FNABs in the Chinese population is limited and there is a need for a comprehensive survey of the distribution of genetic alterations in Chinese thyroid carcinomas. The purpose of this study is to delineate the frequency of the *BRAF*^{V600E} mutation, *RET/PTC* rearrangements, and *RAS* mutations in FNAB samples in the Chinese population, and to evaluate the diagnostic value of molecular testing as an adjunctive diagnostic tool along with traditional cytological analysis in a larger prospective study.

Materials and methods

From September 2012 to December 2013, a total of 314 patients with thyroid nodules were consecutively enrolled at the Department of Endocrinology, The First Affiliated Hospital of Xi'an Jiaotong University Health Science Center. All patients provided informed consent, and the study was approved by the Institutional Review Boards. All of these patients underwent fine-needle aspiration by an experienced endocrinologist under ultrasound-guidance. According to the Bethesda System for Reporting Thyroid Cytology, cytological evaluation by an experienced cytopathologist (BY.S) of FNAB slides revealed 104 conventional PTCs, 13 suspicious for PTC, 52 indeterminate (including atypia of undetermined significance and follicular neoplasm or suspicious for a follicular neoplasm), 108 benign follicular nodules and 37 Hashimoto's disease nodules.

DNA and RNA extraction from fresh FNAB samples

The aspirated material was obtained from a nodule in 3 to 4 passes with a 23-gauge needle. The harvested material was divided into two parts: one part was smeared on a glass slide for cytological evaluation; and the remaining material in the needle plus the needle washing was collected into a 1.5 ml Eppendorf tube containing 1 ml TRI reagent (Invitrogen, Carlsbad, CA) for DNA and RNA isolation following the manufacturer's protocol. The quantity of the isolated DNA and RNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

BRAF^{V600E} *mutation and RAS mutation detection by pyrosequencing*

Samples with sufficient DNA were tested for point mutations in *BRAF*^{V600E} mutation, *NRAS* (codon 61), *KRAS* (codon 12/13), and *KRAS* (codon 61) mutations using a pyrosequencing assay. A total of 25-μl polymerase chain reaction (PCR) mix contained forward and reverse primers (each 0.2-μM), dNTP (0.5-μl), $MgCl_2$ (1.5-mM), 1× PCR buffer, Taq polymerase (0.2 IU) (Life Technologies™, Carlsbad, CA, USA), and 50–100 ng DNA extraction. PCR was performed as follows: 95 °C for 5 min, 35 cycles consisting of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and followed by 10 min extension at 72 °C. The PCR products were electrophoresed in a 1.5% of agarose gel to confirm the successful amplification of the PCR products. Biotinylated PCR products (20-μl) were immobilized on streptavidin-coated Sepharose beads (GE