Original Article

Value of miRs in fine-needle aspiration biopsy of cervical lymph node as marker for the detection of metastasis in patients with papillary thyroid cancer after total thyroidectomy

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Abstract: Background: The presence of metastatic disease in cervical lymph nodes in patients with papillary thyroid cancer after total thyroidectomy is a very important determinant in therapy choice and prognosis, with great impact in overall survival. Frequently, routine lymph node staging cannot detect occult metastases and the post-surgical histologic evaluation of resected lymph nodes is not sensitive in detecting small metastatic deposits. Molecular markers based on tissue-specific microRNA expression are alternative accurate diagnostic markers. Herein, we evaluated the feasibility of using the expression of microRNAs to detect metastatic cells in fine-needle aspiration (FNA) biopsies of patients with papillary thyroid cancer after total thyroidectomy. Methods: miR-124, miR-221 and miR-146 expression was detected by qRT-PCR in 668 FNA biopsies (481 metastatic LNs, 163 benign LNs and the 24 suspicious LNs). The accuracy of the markers in identifying metastatic samples was assessed through the analysis of sensitivity, specificity, accuracy, negative predictive value, positive predictive value, and area under the curve values. Results: miR-221 and miR-146 were highly expressed in metastatic lymph nodes and miR-124 was lowly expressed in metastatic lymph nodes. Additionally, miR-146 showed highest accuracy when FNA samples were examined. Conclusions: The high accuracy of miR-124, miR-221 and miR-146, especially for miR-146 warrant these microRNAs as diagnostic markers of neck metastases in patients with papillary thyroid cancer after total thyroidectomy. These can be evaluated in FNA biopsies collected at different time-points such as patient follow-up. These markers can be useful in a clinical setting in the management of patients with papillary thyroid cancer after total thyroidectomy from initial disease staging and therapy planning to patient surveillance.

Keywords: Diagnostic markers, fine-needle aspiration biopsies, papillary thyroid cancer, MicroRNAs, miR-124, miR-221 and miR-146, neck lymph nodes metastases

Introduction

Papillary thyroid carcinoma (PTC) usually has a good prognosis with indolent course. However, loco-regional recurrence is not negligible, ranging from 5-20% in patients who undergo surgery for PTC [1, 2]. The most frequently involved site of loco-regional metastasis of PTC is the neck, and the incidence of lymph node metastasis (LNM) is relatively high with values reported from 3.1% to 28.9%, which can influence the prognosis [3]. Therefore, several methods are generally used to detect metastases or recurrences of PTC, such as diagnostic whole body scan, neck ultrasonography (US) and serum thyroglobulin (Tg) measurements [4, 5]. Recently, fine-needle aspiration biopsy cytology (FNAB-C) represents the gold standard technique for the detection of cervical lymph node (CLN) metastasis [6]. However, FNAB-C relies on the experience and ability of the cytopathologist, and may be a challenging diagnostic category as CLN could harbor metastasis from a multiplicity of extrathyroidal malignancies or be affected by several non-tumoral diseases [6-8].

It is now accepted that the sensitivity of detecting PTC in lymph node FNA-C is increased by measuring thyroglobulin in needle washouts from FNA biopsy (FNA-Tg) [9, 10]. However, it...
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is very difficult to determine a specific Tg threshold concentration because various kits are used worldwide and also the techniques and volumes used for syringe washout differ considerably.

microRNAs (miRs) constitute a class of endogenous small non-coding RNA fragments (18-24 nucleotides) that regulate gene expression. The biogenesis of miRs begins with transcription as long double-stranded primary transcripts that are subsequently converted into a precursor of ~70 nucleotides, which is finally cleaved in the cytoplasm into the 22-nucleotide double-stranded miR [11].

miRs play a significant role in cancer and regulate major processes such as proliferation, differentiation, and cell death. To date, numerous cases of miR dysregulation or upregulation have been identified as potential contributors to several malignancies [12-14]. He et al. [14] has reported that three miRs (miR-146, miR-221, and miR-222) showed dramatic overexpression, with 11-fold to 19-fold higher level in PTC tumors than in adjacent unaffected thyroid tissue. Chen et al. [15] identified significant upregulation of miR-124, miR-146, miR-221, and miR-222 in PTC samples obtained by both FFPE and ex vivo FNAB, and that miR-146 could potentially serve as a marker for diagnosing PTC using both FNAB and surgical pathology specimens. Recently, a panel of six miRs was used (miR-21, miR-31, miR-146b, miR-187, miR-221, and miR-222) on a sample of 27 ex vivo FNABs, with an accuracy of 98% for predicting PTC [16]. The values for FTC are not as high, and Vriens et al. [17] used miR-100, miR-125b, miR-138, and miR-768-3p with an accuracy of 71% for follicular neoplasms and 98% for Hürthle cell neoplasms. Recently in a preliminary study, residual cells left in the FNAB needle were used to diagnose malignancy in intermediate cytology results with an accuracy of 90% [18].

The purpose of this study was to determine whether miRs measurements in FNAB of lymph node may be used to predict the likelihood of metastatic lymph nodes.

Materials and methods

Patients

During March 2007 to Jul 2015 period, we retrospectively reviewed the data of 7783 patients who underwent thyroidectomy for the treatment of PTC. We confined this study to patients who underwent total thyroidectomy and LN dissection including central or lateral neck dissection. We excluded patients who did not receive neck dissection or total thyroidectomy, and had distant metastasis before operation. Finally, 1744 patients were enrolled. Of the 1744 patients, FNAB-miR-124, miR-221 and 146 measurements of suspected cervical LN metastasis were performed in 437 consecutive PTC patients (668 FNA biopsies) at the department of diagnostic ultrasound, the affiliated hospital of Qingdao University. The study was approved by the Institutional Review Board of the affiliated hospital of Qingdao university.

Fine-needle aspiration biopsies processing and RNA purification

The material collected through FNA from lymph nodes in 437 consecutive PTC patients after total thyroidectomy. The lymph nodes were used to perform a smear, and the slides were stained to enable the cytological diagnostic of the lymph nodes as ‘positive’ or ‘negative’ for the presence of metastatic cells. The remaining material on the needle was washed in 200 μL of sterile saline solution and ethylenediaminetetraacetic acid, snap frozen, and stored at -80°C until RNA extraction. The extraction of total RNA from FNA samples was performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as the manufacture's instruction. Total RNA was quantified in the Qubit fluorometer (Invitrogen) and stored at -80°C until use.

Quantitative real-time PCR (qRT-PCR) for miRs measurement

Total nucleic acids were isolated from FNA samples using magnetic glass particles (Roche) according to the manufacturer’s protocol. MiRNA was isolated using amiRN easy kit (Qiagen) from the total RNA above. Single-stranded cDNA was synthesized using Taqman Fast System and reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. All the primers (miR-124, miR-221 and miR-146) were synthesized by Sangon.com (Shanghai, China). Data were analyzed with the aid of ABI Prism 7700 SDS software (Applied Biosystems).
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miRNA expression levels in lymph nodes samples were calculated. miRNA expression levels in metastatic lymph nodes compared to those of no metastatic lymph nodes. Relative expression levels were analyzed using the cycle threshold (Ct) method. The expression level of each miRNA was normalized to that of U6sn RNA (an internal control). Control amplification of the small endogenous RNA U6 (a housekeeping gene) was performed on all samples. The post amplification thresholds for all samples were set to be identical, to allow the Ct values of each miRNA to be compared. Mean Ct values were calculated for each specimen and normalized to those of RNA U6 in the same samples. Thus: miRNA expression levels were normalized by subtracting the Ct value of the U6sn RNA internal control from that of each miRNA (Ct miRNA minus Ct U6sn).

Statistical analyses

The data were expressed as means ± SE. The Mann-Whitney U test was used to compare miRNA expression levels between benign and malignant samples. The area under the ROC curve (AUC) was able to identify optimal sensitivity and specificity levels to distinguish metastatic samples from non-metastatic ones. Sensitivity, specificity, accuracy, and positive and negative predictive values (PPV and NPV) of FNAB-miRs in distinguishing metastatic from non-metastatic samples were also calculated along with 95% confidence intervals (95% CI). All statistical analyses were performed with SPSS.17.0. Data with a $P<0.05$ were considered significant.

Results

Identification of metastatic cell deposits in FNA lymph node samples

Overall, 668 FNA biopsies were collected from lymph nodes of 437 patients. The final diagnosis of the 668 LNs was established by pathological examination. 481 (72%) LNs were finally diagnosed as metastatic LNs (Figure 1A), and 163 (24.4%) were diagnosed as benign LNs (Figure 1B).

Quantitative analysis of miRs in FNA samples

We analyzed the expression of miR-124, miR-221 and miR-146 molecules in 481 (72%) met-
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Figure 2. Box plots of miRNA expression levels in metastatic LNs and benign LNs. The lines inside the boxes indicate the medians.

Figure 3. Receiver operating characteristic curves obtained for microRNAs miR-221, miR-124, and miR-146 in the FNA samples.

Clear and 163 (24.4%) benign LNs by quantitative real-time PCR (qRT-PCR). The distribution of the miRNA levels in the two groups are shown in Figure 2A-C. miR-221 and miR-146 miRNA levels were markedly higher and miR-124 levels were lower in metastatic LNs when compared with the benign LNs. miR-124, miR-221 and miR-146 expression showed sig-
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Significant difference between metastatic LNs and benign LNs (P<0.001, respectively).

**Predictive value of the levels of miRNA in lymph nodes**

To evaluate the diagnostic values of miR-124, miR-221 and miR-146 expression, ROC curve analysis was performed in 481 metastatic LNs and 163 benign LNs. A comparison of the metastatic LNs with those of benign LNs indicated that the levels of miR-124 had an AUC of 0.856 [95% confidence interval (CI)=0.764-0.928] (P=0.001), miR-221 an AUC of 0.863 [95% CI=0.712-0.917] (P=0.016) and miR-146 an AUC of 0.956 [95% CI=0.91-1.0] (P<0.001) (Figure 3A-C). The cutoff value of miR-124, 221 and 146 for metastatic LNs was 0.367, 1.39 and 9.76, their sensitivity and specificity were 89.2% and 65.4%, 78.3% and 67.8%, 94.6% and 83.4%, respectively. The positive predictive value (PPV), negative predictive value (NPV), and overall accuracy was 83.4%, 93.3% and 74.6%, 74.5%, 82.6% and 69.4%, 93%, 89% and 82% for miR-124, miR-221 and miR-146, respectively. miR-124, miR-221 and miR-146 expression was also detected in the 24 suspicious cases. All of them have low value, but there was no statistical difference compared to the benign LNs (data not shown).

**Discussion**

Cytological evaluation of FNAB represents the gold standard technique for the diagnosis of CLN suspected to harbor metastatic disease from thyroid cancer as well as from other primary tumors. The technique accuracy, highly dependent on the experience and ability of the cytopathologist, has been reported to vary from 73% to 94% [19-21]. Over the last years, following clinical evidence showing that FNAB-Tg in fine-needle washout improves the accuracy of FNAB-C in the evaluation of CLN metastases of DTC, it has been recommended the routine association of FNAB-Tg with FNAB-C in the preoperative diagnosis of suspicious CLN [22, 23]. Although the sensitivity of detecting DTC in lymph node FNA-C is increased by measuring thyroglobulin in needle washouts from FNA biopsy (FNA-Tg), the diagnostic threshold has not been well established. Recent studies have reported interference from Tg antibodies (Tg Ab) leading to low or false-negative results [23, 24]. To overcome this problem, molecular detection of metastases seems to be one of the most promising methods for definitive lymph node evaluation. Through this approach, metastatic deposits in lymph nodes can be detected in a more sensitive, accurate, and less time-consuming manner. Toward this end, the identification of molecular markers capable of detecting the presence of metastatic cells in a background of lymphatic cells is mandatory.

Lastly, Shen et al. [25] used archived FNAB slides to extract RNA and evaluate the malignancy potential of those determined “follicular lesion of unknown significance” with a panel of seven miRs (miR-30d, miR-138, miR-146b, miR-187, miR-197, miR-221, miR-302c, and miR-346). The overall diagnostic accuracy for these challenging FNAB samples was 85%. Recently in a preliminary study, residual cells left in the FNAB needle were used to diagnose malignancy in intermediate cytology results with an accuracy of 90% [26]. Another diagnostic aspect that has been studied is the use of miRs to predict tumor aggressiveness [27]. miR-146b, miR-221, and miR-222 have found correctly to identify PTC [28]. We recently have found low miR-124 expression in PTC had more lymph node metastasis (Inpressed).

In the present study, we determine whether miR-124, miR-221 and miR-146 measurements in FNAB of lymph node may be used to predict the likelihood of metastatic lymph nodes in patients with papillary thyroid cancer after total thyroidectomy. The results showed that miR-221 and miR-146 mRNA levels were markedly higher and miR-124 levels were lower in metastatic LNs when compared with the benign LNs. miR-124, miR-221 and miR-146 expression showed significant difference between metastatic LNs and benign LNs. miR-124, miR-221 and miR-146 were able to detect the presence of metastatic deposits. This highlights the high sensitivity of these markers in detecting epithelial cells in a lymphoid tissue background, allowing the correct detection of metastases in entire lymph nodes and avoiding false negative results related to partial lymph node assessment. Moreover, a methodology for automated qRT-PCR-based gene expression analysis in an average time of 35 minutes has
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been described recently [29]. Taken collectively, these results suggest the feasibility of performing microRNA marker analysis in lymph nodes to assist the surgeon in deciding the best approach to be adopted in the neck treatment.

This study showed that the comparison between cytology assessment, histology examination, and molecular analysis presented high levels of agreement in detecting metastatic cells in lymph nodes. Most FNA samples harboring metastases (according to pathological evaluation) could be correctly identified through the use of these molecular markers. However, miR-146 has the highest sensitivity, specificity and overall accuracy among the three miRs. The 24 suspicious cases have low miRs, which was due to sampling errors represented by the absence of metastatic cells in the FNA specimen. Since metastatic cells were not aspirated, they were not present in the cytological smear and consequently were also absent in the sample obtained from washing the aspiration needle.

Even though the molecular approach did not show a significant improvement in accuracy when compared to the cytological assessment, we believe that its use is warranted given that, unlike molecular assessment, the accuracy of cytological assessments is highly dependent on the quality of the preparation and the diagnostic skills of the cytopathologist. The results obtained with the analysis of FNA samples indicate a high specificity and sensitivity of miR-124, miR-221 and miR-146, especially for miR-146 to detect the presence of metastatic cells in the FNA biopsy samples, suggesting their role in a future pre-treatment or follow-up test of suspicious lymph nodes in patients with papillary thyroid cancer after total thyroidectomy.

To the best of our knowledge, this is the first study to demonstrate the usefulness of miR-124, miR-221 and miR-146 expression as a sensitive, specific, and accurate molecular approach for the diagnosis of cervical lymph node metastases in patients with papillary thyroid cancer after total thyroidectomy. Our results suggest that the evaluation of miR-124, miR-221 and miR-146 expression, especially for miR-146 expression, could be an important tool for the management of patients with papillary thyroid cancer after total thyroidectomy, assisting in the stratification of patients that may harbor neck metastases, aiding in therapy planning and patient surveillance, ultimately contributing to an improvement in quality of life and survival rates.

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Disclosure of conflict of interest

None.

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