RESEARCH ARTICLE

Serum Oncofetal Fibronectin (onfFN) mRNA in Differentiated Thyroid Carcinoma (DTC): Large Overlap between Disease-Free and Metastatic Patients

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Abstract

Aim: This study assessed if onfFN mRNA in the peripheral blood of patients with DTC can identify individuals with metastatic disease. Methods: Comparison of onfFN mRNA was made among 3 groups: disease-free, lymph node metastasis, and distant metastasis using real-time RT-PCR on 5 ml blood samples from each DTC patient. Results: Fifty-one patients were included: 30 (59%) were disease-free; 7 (13.7%) had lymph node metastasis; and 14 (27.5%) had distant metastasis. OnfFN mRNA levels in the 3 groups were significantly different (P=0.001) but with a large overlap and the expression being highest in the disease-free group. Subgroup analysis of the metastatic groups did not show any effect of age, cell type, and serum TSH, Tg, and antiTg on onfFN mRNA. The within-run and between-run root mean square coefficients of variations were <2%. Conclusion: OnfFN mRNA in patients with DTC cannot identify those with metastatic disease.

Keywords: Oncofetal fibronectin - mRNA - real-time RT-PCR - differentiated thyroid carcinoma - metastasis

Asian Pacific J Cancer Prev, **13**, 4203-4208

Introduction

Follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC) are considered to be differentiated thyroid carcinoma (DTC), the most common endocrine malignancy. Its incidence has been reported to be increasing in Europe (Leenhardt et al., 2004; Burgess, 2006; Rego-Iraeta et al., 2009) and the USA (Davies, 2006) although this may be attributable to more frequent use of medical imaging, leading to an increased detection rate of small, subclinical tumours. The principles of treatment involve removal of all cancerous cells by thyroidectomy and then ablation of residual normal thyroid tissue in an effort to decrease the risk for recurrent locoregional disease. After thyroidectomy and radioiodine ablation, serum thyroglobulin (Tg) becomes a sensitive and specific marker for the presence of recurrent or metastatic disease. The role of serum Tg determination has been well established as an early indicator of recurrence of DTC (Cooper et al., 2009).

However, the sensitivity of Tg detection is highest under the stimulation of thyroid stimulating hormone (TSH) (Eustatia-Rutten et al., 2004), which can be achieved either by withdrawal of thyroxin or by using recombinant human TSH. Using the former method, the patients have to suffer from hypothyroid condition, potentially promoting the growth of thyroid cancerous cells whereas the latter is costly and unaffordable by most patients in developing countries.

In addition, antithyroglobulin antibodies (antiTg) are detected in the sera of 25% of patients with DTC (Spencer et al., 1998) and 10% of general population (Hollowell et al., 2002). In the presence of antiTg, serum Tg level determination may be interfered, leading to false negative results (Schaadt et al., 1995; Spencer et al., 1998; Rosario et al., 2004). Recurrence or metastasis was confirmed in 39%-49% of those with elevated antiTg and undetectable Tg (Rubello et al., 1992; Hjiyiannakis et al., 1999; Chung et al., 2002). That is, the remaining ~60% of the patients have uncertain disease status. Therefore, these patients must be monitored with neck ultrasonography and periodical ^{131}I total body scan, (Pacini et al., 2006) resulting in higher health expenditure, higher patient radiation dose and inconvenience. Measurement of Tg mRNA in the blood has a potential of circumventing this problem as it has been suggested as a means of detecting the presence of thyroid cells; nevertheless, the reported results have been inconsistent posing the question of its specificity (Cooper et al., 2009).

In search of a more promising tumour marker, a

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number of potential tumour markers including the mRNA of Tg, thyroid-stimulating hormone receptor (TSH-R), cytokeratin 19 (CK19), human telomerase reverse transcriptase (hTERT), and oncofetal fibronectin (onfFN) transcripts in leukocytes, as well as in PTCs and FTCs have been studied (Hesse et al., 2005). Interestingly, onfFN transcripts were detected in peripheral blood samples of 6 out of 9 patients with known DTC metastases, suggesting the possible role of onfFN as a marker. Moreover, using real-time PCR for monitoring onfFN mRNA was proposed to be superior to other potential tumour markers for detecting minimal residual disease in DTC with regard to both assay sensitivity and specificity.

The objective of this study was to use real-time PCR to assess the performance of the OnfFN mRNA in the peripheral blood of patients with DTC in identifying those with evidence of metastatic disease.

Materials and Methods

The Institutional Review Board of Ramathibodi Hospital approved the study protocol. Patients with DTC (either PTC or FTC) underwent the standard protocol of treatment which included total thyroidectomy, radioiodine ablation, and TSH suppression with thyroxin. Then the patients were followed up at 3-6 months' intervals with physical examination, serum stimulated Tg and antiTg. A diagnostic ^{131}I total body scan with either SPECT-CT fusion or neck ultrasonography was performed 6-12 months after radioiodine ablation to ascertain the success of the ablation of thyroid remnant and to detect lymph or distant metastasis. Those with evidence of residual or recurrent disease would be treated by surgery, if feasible, or radioiodine treatment. Other treatment options, such as radiofrequency ablation and external radiation therapy, were considered for the lesions that could not be treated surgically and did not take up radioiodine.

The patients, whose disease statuses have been ascertained, were invited to take part in the study. Those who agreed to give written inform consent were included. They formed 3 groups according to disease statuses: disease-free patients, patients with lymph node metastasis, and patients with distant metastasis. For the latter 2 groups, their lesions were documented on either ^{131}I total body scan or radiographic examinations by computed tomography. The patients in the disease-free group complied with the ATA 2009 criteria (Cooper et al., 2009). They were asymptomatic and had no evidence of disease on clinical examination and imaging (no uptake outside the thyroid bed on the initial post-treatment ^{131}I total body scan and negative recent diagnostic ^{131}I total body scan with negative neck ultrasonography or SPECT-CT). In addition, they had stimulated Tg of less than 1 ng/ml as well as low antiTg. A peripheral blood sample of 5 ml was collected from each patient into an EDTA-tube for the analysis of OnfFN mRNA, TSH, Tg, and antiTg levels.

^{131}I total body scan

To document the success of ablation or to detect the lesions, ^{131}I total body scan was performed with 74 MBq of ^{131}I given orally after 6 weeks of L-T4 withdrawal and when serum TSH was at least 30 IU/ml. At 48 hours, anterior and posterior views of total body were acquired over 30 minutes using a dual head Genesys gamma camera with a high-energy collimator (Philips). The scan was considered abnormal when there were one or more areas of increased uptake at the thyroid bed, in the liver, or outside the normal distribution. Two experienced nuclear medicine physicians assessed the scans. To avoid bias the scans were initially assessed without the knowledge of the clinical condition and subsequently with all the information provided. Any disagreements were resolved with consensus. When the Tg (obtained at the same time with TSH) was at least 1 ng/ml or the images were negative or equivocal, SPECT of the neck and chest was performed with C_T fusion. Otherwise, the patient was sent for an ultrasonography of the neck.

Analysis of OnfFN mRNA

Reverse transcription and semi-quantitative real-time PCR: Total RNA was freshly prepared from the collected blood using QIAamp® RNA Blood mini kit (Qiagen, Germany) according to the manufacturer's instruction. Reverse transcription (RT) was performed using 2 μg of total RNA in a RT mixture containing 50mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 20 mM dNTPs, 0.4 mM Dithiothreitol, 200 U SuperScript® III Reverse Transcriptase (Invitrogen, USA) and 75 mM oligo $(dT)_{18}$ in a total volume of 20 ml at 50 °C for 60 minutes.

One microliter of the first-strand cDNA was used for semi-quantitative real-time polymerase chain reaction (real-time PCR) to determine the onfFN transcript levels using primers onfFN-F [5'-GCTTCCTGGCACTTCTGG-3'] and onfFN-R [5'-CATTCGGCGGGTATGGTC-3'] and the internal control gene β-actin, using primer β-Actin-F [5'-CTCTTCCAGCCTTCCTTCCT-3'] and β-actin-R [5'-AGCACTGTGTTGGCGTACAG-3']. All the primers were from Operon, Singapore. Real-time PCR was carried in the 7500 Real-time PCR system (Applied Biosystems, USA) using the Power SYBR Green PCR Master Mix (Applied Biosystems, USA). The reaction was carried out in 1X SYBR green master mix containing 100 μg of first strand cDNA, 20 μM each forward and reverse primers. The condition for the real-time PCR was conducted as followed: 95°C for 10 min and 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 70°C for 30 sec. All experiments were performed in triplicate.

Quantitative and data analysis

The onfFN mRNA in the peripheral blood was quantified using the comparative threshold cycle (CT) method (Livak, 2001; Schmittgen et al., 2008). C_T was defined as the cycle number at which the amplification crosses a fixed threshold above baseline. Data analysis was conducted by using ABI prism 7500 SDS software (Applied Biosystems). Briefly, the ΔC_T from each sample was calculated from the subtraction of an average onfFN C_T by an average β-actin C_T. The number of fold of onfFN mRNA ($2^{-\Delta CT}$) was determined.

Determination of TSH, Tg, and AntiTg levels

The serum TSH was determined with an immuno-

chemiluminescence assay using Architech System (Abbott, Illinois, USA), which has a functional sensitivity of 0.0038 μIU/ml and coefficient of variation (CV) of 1.9-5.3%. Both serum Tg and AntiTg levels were measured using electrochemiluminescence immunoassay on Elecsys and cobas® immunoassay analyzers (Roche Disgnostics GmbH, Penzberg, Germany). The assay for serum Tg had a detection limit of 0.1 ng/ml and CV of 1.8-3.6%, whereas that for AntiTg had a detection limit of 10 IU/ml and CV of 1.3-4.9%.

Evaluation of the precision

To evaluate the precision of the assay, within-run and between-run variation were determined.

Within-run variation: Within-run variation of onfFN C_T and β-actin C_T were determined by calculating root mean square (RMS) of the coefficient of variation (CV) by the following steps. Firstly, the mean and the SD of the triplicate C_T of each patient sample were calculated. Secondly, CV (SD/mean) of each patient was obtained. Finally, all patients' CVs within each run were summed up, averaged and the square root was calculated to give overall within-run CV (RMS CV).

Between-run variation: Assuming minimal variation in both positive and negative controls in each run, between-run variation was calculated in the same way. The values of mean, SD, and CV of the triplicate C_T of each control were calculated. Similarly, all CVs were summed up, averaged and the square root calculated to give between-run variation.

Statistical analysis

Data were described using medians (range) and numbers (percentage) for continuous and categorical data, respectively. The median onfFN mRNA values between the 3 groups were compared using Kruskal-Wallis test. Subgroup analysis within the 2 disease groups (combination of patients with lymph node and distant metastasis) was performed to further assess factors potentially associated with the onfFN mRNA using Mann Whitney test. These factors included age, cell type, serum TSH, antiTg or Tg. Both were performed using STATA version 11.0. The P value less than 0.05 was considered as statistically significant.

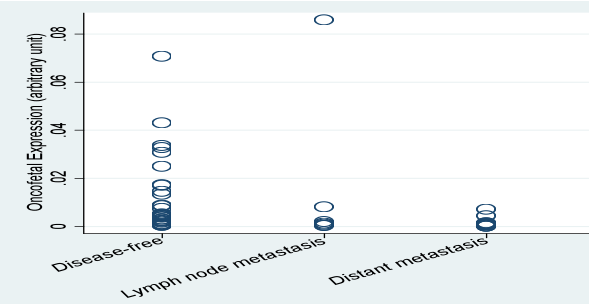

Figure 1. Comparisons of the OnfFN mRNA Levels among the 3 Groups of Patients.

Results

Patients

During July 2007-July 2008, patients who met the inclusion criteria were enrolled when they came for a follow-up at Nuclear Medicine Unit, Ramathibodi Hospital. Fifty-one patients, aged 39 (11, 80) years, agreed to participate; 10 (19.6%) were male. There were 11 (21.6%) patients with FTC. Thirty (59%) patients were disease-free while 7 (13.7%) had lymph node metastasis, and 14 (27.5%) had distant metastasis. Eight patients had serum TSH of at least 30 mU/ml because they were enrolled right after diagnostic or post-therapeutic ^{131}I TBS while the rest were on LT4 for TSH suppression. The patient characteristics and the onfFN mRNA are summarised in Table 1.

Among the distant metastasis group, 7 had isolated lung metastasis, 5 had isolated bone metastasis, and 2 had both lung and bone metastases. Six out of 7 patients with isolated lung metastasis, 3 out of 5 patients with isolated bone metastasis, and 1 out of 2 patients with both lung and bone metastases were due to PTC. All patients with lymph node metastasis were due to PTC. Six patients had stopped levo-thyroxin for either ^{131}I treatment or diagnostic ^{131}I total body scan.

Among these 3 groups of patients, the onfFN mRNA was significantly shown to be highest in the disease-free group and lowest in the distant metastasis group (P=0.001). The graph showing onfFN mRNA is shown in Figure 1. Median Tg, antiTg, and TSH levels were also significantly higher in the distant metastasis than the other two groups.

Subgroup analysis

Among patients with active disease (lymph node and distant metastasis), the median onfFN mRNA between

Table 1. Patient Characteristics and onfFN mRNA

Patient Characteristics	Disease-Free	Lymph Node Metastasis	Distant Metastasis	P value
Number of patients[1] (%)	30 (59)	7 (13.7)	14 (27.5)	
Male[1] (%)	4 (13.3)	1 (14.3)	5 (35.7)	0.21
Age[2] (years)	37 (19, 68)	34 (11, 80)	49 (30, 70)	0.36
FTC:PTC	7:23	0:07	4:10	0.31
I-131 cumulative dose[2] (GBq)	5.6 (5.6, 11.1)	8.1 (2.4, 16.2)	14.2 (3.5, 31.1)	<0.001*
TSH[2] (mU/ml)	0.01 (0.01, 100)	0.16 (0.02, 100)	1.72 ((0.01, 100)	0.043*
Tg[2] (ng/ml)	0.1 (0.1, 0.735)	131.7 (0.6, 2712)	262.5 (3.3, 100000)	<0.001*
AntiTg[2] (IU/ml)	11.8 (10.0, 32.79)	12.9 (12.9, 790.9)	20.7 (10.0, 4000)	0.018*
Follow-up[2] (months)	39.0 (5.6,47.4)	33.3 (18.1, 33.7)	31.8 (2.8, 34.3)	<0.001*
OnfFN mRNA[2] (arbitrary unit)	0.0048 (0.0006, 0.0710)	0.0014 (0.0004, 0.0858)	0.0008 (0.0003, 0.007)	0.001*

[1]Numbers (percentage), [2]Medians (minimum, maximum)

Table 2. Subgroup Analysis of the Effect of Clinical Factors on the mRNA of onfFN in the Disease Groups (Combination of Lymph Node Metastasis and Distant Metastasis)

Factors		N	Oncofetal MRNA Median (min, max)	P value
Disease location	Lymph node metastasis	7	0.0014 (0.0004, 0.0858)	0.26
	Distant metastasis	14	0.0008 (0.0003, 0.0007)	
Age (years)	<45	10	0.0012 (0.0003, 0.0858)	0.67
	>45	11	0.0009 (0.0003, 0.0083)	
Cell-type	PTC	17	0.0012 (0.0003, 0.0858)	0.15
	FTC	4	0.0008 (0.0003, 0.0008)	
Serum TSH	Low (<0.5 mU/ml)	12	0.0014 (0.0003, 0.0858)	0.26
	High (≥30 mU/ml)	6	0.0008 (0.0003, 0.0072)	
AntiTg	Low (<24 IU/ml)	12	0.0010 (0.0003, 0.0858)	0.48
	High (>148 IU/ml)	6	0.0009 (0.0003, 0.0045)	
Tg	Low (<10 ng/ml)	6	0.0011 (0.0003, 0.0083)	0.88
	High (Tg >50 ng/ml)	15	0.0001 (0.0003, 0.0858)	

*AntiTg, antithyroglobulin antibodies, FTC, follicular thyroid carcinoma; n, number of patients; PTC, papillary thyroid carcinoma; Tg, thyroglobulin; TSH, thyroid stimulating hormone

Table 3. Within-run Variability from 4 Different Runs Expressed as the Root Mean Square of Coefficient of Variation (RMS CV)

	Run			
Within-run RMS CV (%)	1st	2nd	3rd	4th
onfFN C_T	1.11	0.62	0.34	0.66
beta-actin C_T	1.2	0.59	0.7	0.43

groups of several potential factors (including age, cell type, serum TSH level, serum anti Tg level, and serum Tg) were compared (Table 2). The median onfFN mRNA was higher in the lymph node metastasis than distant metastasis but this did not reach to statistically significant (0.0014 versus 0.0008 arbitrary units, P=0.263). Moreover, the levels of onfFN mRNA in PTC patients were not significantly different from those with FTC (0.0012 versus 0.0009 arbitrary units, P=0.152). There was an inverse association between onfFN mRNAs and Tg, antiTg, and TSH level, i.e., the lower Tg, anti Tg, and THS levels, the higher onfFN mRNAs, see Table 2.

Precision

The within-run RMS CVs (Table 3.) were 0.34%-1.11% and 0.43%-1.20% for onfFN C_T and β-actin CT, respectively.

The between-run RMS CV for onfFN C_T was 1.71%.

Discussion

Based on the use of OnfFN mRNA in identifying metastatic DTC by Hesse et al (Hesse et al., 2005), we applied to assess if the onfFN mRNA in the peripheral blood of the patients with metastatic DTC can be used to differentiate between the active and disease-free groups. Generally, the patients were considered disease-free after total thyroidectomy and radioiodine ablation. Since we did not aim to study the performance of ^{131}I total body scan, the nuclear medicine physicians were not blinded to clinical information as the clinical statuses needed to be accurate before enrolling the patients into the study. There was no change in the clinical status occurred during the follow-up period (median follow-up time of 33.8 months) in the disease-free group. The comparative C_T method used for quantifying the mRNA of onfFN in this study is an acceptable technique (Chang et al., 2002; Schmittgen, 2008; Ward et al., 2009). The between-run and within-run variations were less than 2%, suggesting reliability of the procedure. However, the onfFN mRNA was not higher in the groups with metastasis. On the contrary, it was statistically highest in the disease-free group and lowest in the distant metastasis group (P=0.001).

Our observations were indeed similar to the subsequent work by the same group (Wehmeier et al., 2010) 27 in that for the disease-free group, the onfFN expression range was wide and the maximum value was higher than the metastatic groups. Their explanation was that their disease-free patients were perhaps in fact harboring disease that was not detected by their follow-up scheme and that the onfFN mRNA was very sensitive for disease detection. Using their proposed cutoff point, half of their disease-free patients may need to undergo further investigations in addition to their usual follow-up scheme to document the disease status. However, they did not state explicitly how they defined disease-free patients; presumably they all had negative radioiodine total body scan and neck ultrasonography. This raises the question on cost-effectiveness of using the onfFN expression. It has been reported that the cultured fibroblasts derived from normal thyroid tissues were shown to express a high copy number of onfFN mRNA (Takano et al., 2000). The expression of the cultured fibroblast was even higher than most of the PTC fine needle aspiration biopsy samples. Hence, the findings in the peripheral blood may be affected by factors other than the presence of active disease alone.

Despite the lack of statistical significance, a "strong trend" to higher expression rates of onfFN-mRNA in patients with metastases was asserted (Wehmeier et al., 2010). Their metastatic group was defined by having serum Tg > 2 ng/ml regardless of negative imaging results. The fact that their scatter plot of onfFN mRNA showed one very high outlier in the group with metastasis could explain their so-called strong trend. Without this one outlier, the mean and the standard deviation of onfFN mRNA of this group would have been much lower.

The main differences between their work and ours were inclusion criteria and patient classification. To assess the discrimination performance of the onfFN mRNA in the best-case scenario, only patients with unequivocal evidence to determine the disease status were included. The reason for not including the patients with the serum Tg level of > 2 ng/ml without imaging evidence of metastasis was that a single Tg determination can be fluctuating with a reported within-assay coefficient variation of at least 5-7% (Ferrari et al., 2004). Moreover, the clinical significance of minimally detectable Tg levels is unclear, especially if only detected following TSH stimulation (Cooper et al.,2009). Seventy per cent of patients with positive Tg and negative diagnostic total body scan remained disease free during 8 years of follow-up (Alzahrani et al., 2002). In addition, benign sources of Tg secretion have been proposed (Zanotti-Fregonara et al., 2010). These sources

may be foci of radio-resistant ectopic thyroid tissue or a TSH-stimulated thymus. To classify a patient as having metastasis based solely on the basis of a single observation of serum Tg level of > 2 ng/ml without further validation may be inaccurate. Hence, our metastatic groups were those who had imaging evidence on either ^{131}I total body scan or computed tomography in addition of having the serum Tg level of > 2 ng/ml. Even with a loose criterion of metastasis, the metastatic group of constituted only 8% of the patients in the study of Wehmeier et al. (2010); while in ours the combined metastatic groups constituted 41%, or the group with distant metastasis alone 27%.

Also, the patients with ^{131}I uptake confined to the thyroid bed were not included because it was not possible to precisely differentiate normal residual thyroid from residual tumour when there was no pathological proof and the lesions were frequently too small to characterise on ultrasonography. By including these patients in the work of Wehmeier et al. (2010) 27, their group of local residual disease may comprise those with merely normal residual thyroid. The onfFN expression in this group is likely to be similar to those in the disease-free group, whose range and maximum expression were higher than those with distant metastasis. This finding is in fact similar to ours. The similarity could be more obvious if the local residual disease group and the disease-free group were combined.

Among the 3 groups, the statistically significant difference in the cumulative dose and the level of serum Tg between the disease-free and disease groups was expected because those with the presence of disease typically have higher level of tumour marker and required more ^{131}I treatment. The difference in the level of antiTg may have reached statistical significance if the sample size were larger. The antiTg level in the disease free group was lower because the patients with high antiTg would only be included in the disease groups of this study when there was an evidence of disease clinically or radiographically. On the other hand, when there was no other evidence of disease accompanying high antiTg, the disease statuses were uncertain and the patients were not included.

Age has been included in TNM staging. In the presence of distant metastasis, patients younger than 45 years of age are classified as stage 2 while those older are classified as stage 4. The effects on the onfFN mRNA of age and other clinical factors including cell type, serum level of TSH, Tg, and antiTg were explored among the disease groups. The lack of statistically significant difference could be due to small sample size.

The detectable limit of antiTg in this study was 10 IU/ml. However, there were only 12 of 30 (40%) patients in the disease-free group who had antiTg of less than 10.0 IU/ml. If this was used as a cutoff point to indicate the absence of antibody interference, a large percentage of patients would have to be put on a watchful follow-up scheme and considered having an indeterminate status until either being subsequently proved as having the disease or their antiTg levels have declined to undetectable level. The cutoff point of antiTg by immunochemiluminescence technique in our clinical practice based on our previous report is 50 IU/ml (Sritara et al., 2008). It is the 95th percentile of antiTg level in 140 patients whose antiTg had been undetectable by immunoradiometric assay.

As it was previously reported that the onfFN expression was present only in papillary and anaplastic carcinomas (Takano et al., 1998; Takano et al., 1999; Takano et al., 2007), the inclusion of higher proportion of patients with FTC in our study group could lower the median onfFN expression in the disease groups. However, the maximum onfFN expression in the disease group was higher in PTC than in FTC. Also, there were only 4 FTC out of 21 patients (19.0%) in the disease groups and 7 out of 30 (23.3%) in the disease-free group, therefore, the inclusion proportion of patients with FTC should not account for this indifference. In fact, Hesse et al (Hesse et al., 2005) showed that detectable levels of onfFN were found in the majority of the tissue samples of FTC but only 11% having high expression. Also, a quarter of all PTC tumours only displayed intermediate and low (13%) onfFN signals. Hence, the inclusion of patients with FTC does not explain the lower onfFN expression in the disease groups in our study.

Our limitations include the lack of uniform control of TSH level and a small sample size rendering it unable to assess potential clinical factors associated with the onfFN mRNA. As a result, lower level of onfFN mRNA in patients the disease groups than those in the disease-free group might be due to individual cohort chance, or in fact, the onfFN mRNA itself does not associate with disease progression. Exclusion of the patients with uncertain disease status can overestimate the ability of the test to identify those with disease. However, overestimation has not been the case in this study. To document the usefulness of onfFN mRNA as a tumour marker, further studies should design to evaluate the influence of relevant clinical factors, such as TSH stimulation and the presence of antiTg, in a larger sample size.

In conclusion, onfFN mRNA in the peripheral blood of patients with differentiated thyroid carcinoma cannot identify those with evidence of metastatic disease.

Acknowledgements

The authors thank the Faculty of Medicine Ramathibodi Hospital for funding this study and Mr.Maurice Broughton for manuscript correction.

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